Responses to Drought Stress Modulate the Susceptibility to Plasmopara Viticola in Grapevine

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Research article

Abstract

Climate change will increase the occurrence of plants simultaneously suffering drought and pathogen stress. Although it is well-known that drought can alter the way plants respond to pathogens, the knowledge about the effect of concurrent drought and biotic stress in grapevine is scarce. This is especially true for *Plasmopara viticola*, the causal agent of grapevine downy mildew. This research addresses how vines with different drought tolerance respond to the challenge with *P. viticola*, drought stress or their combination, and how one stress affects the other. An artificial inoculation was performed on two cultivars, exposed to full or deficit irrigation, in the Mediterranean climate of Cyprus. In parallel, leaf disks from these plants were inoculated in controlled conditions. Leaves were sampled at an early infection stage to determine the influence of the single and combined stresses on oxidative parameters, chlorophyll and phytohormones. Under irrigation, the local Cypriot cultivar Xynisteri was more susceptible to *P. viticola* than the drought-sensitive Chardonnay. Drought stress increased their susceptibility in leaves inoculated in controlled conditions. Conversely, both cultivars showed resistance against *P. viticola* when inoculated *in planta* under continued deficit irrigation. Despite their resistance, the pathogen-associated responses in auxin, antioxidant enzyme activity and proline still occurred in these drought-stressed plants. Surprisingly, abscisic acid, rather than the generally implicated jasmonic and salicylic acid, seemed to play a prominent role in this resistance. The irrigation-dependent susceptibility highlights that the changing climate and the practices used to mitigate its effects, may have a profound impact on plant pathogens.

Main Conclusion

Plasmopara viticola cannot successfully infect intact, drought-stressed grapevine. ABA, rather than JA and SA, is implicated in this resistance. Conversely, drought exposure increased the susceptibility of *in vitro* inoculated leaves.

Introduction

The global climate is changing. The ongoing rising temperatures, shifting precipitation patterns and changing frequency of extreme weather events, such as droughts, are putting a significant strain on many ecosystems (IPCC 2018). Many viticultural areas already suffer from seasonal drought and water availability may become the bottleneck of wine production. The Mediterranean, a hotspot for wine production, is one of world's regions most vulnerable to the impact of climate change (IPCC 2013). Global warming is very likely to aggravate soil moisture drying in this region, augmenting the intensity and frequency of the drought episodes (IPCC 2013).

In the Eastern Mediterranean, the island of Cyprus is known for its hot, arid summers. Its mean annual precipitation is projected to decline with more than 20% by midcentury (Chenoweth et al. 2011). Viticulture has played an important role in Cyprus for over 5,500 years (Lentini 2009). In 2017, vineyards covered 5% of the agricultural area (Statistical service of Republic of Cyprus 2016). With only 11% of the

vine area under irrigation (Statistical service of Republic of Cyprus 2016), most indigenous cultivars can be grown without irrigation. Drought tolerance has obviously been an important selection criterion for these local grapevine cultivars. Since the end of the 20th century however, the international market pushed Cyprus towards the use of commercial cultivars. These introduced cultivars require more water and fertilizers because they are not adapted to the less fertile Cypriot soils and the arid conditions (Litskas et al. 2017).

The changing climate will also significantly affect plant pathogens' traits, such as distribution, virulence, abundance and host range (Garrett et al. 2006; Elad and Pertot 2014). This will increase the likelihood of a combined occurrence of drought and pathogen stress, already a very common event (Ramegowda and Senthil-Kumar 2015). The complexity of the interactions between plant and pathogen gains another dimension when exposed to drought stress, resulting in a new state of stress (Gupta and Senthil-Kumar 2017). Because of overlap and crosstalk between the responses to the individual stresses, this new stress will instigate tailored responses, customized to this specific stress combination (Atkinson and Urwin 2012). Thus, the response to the concurrent challenge cannot simply be interpolated from the independent stress response (Fujita et al. 2006; Atkinson and Urwin 2012; Kissoudis et al. 2014; Ramegowda and Senthil-Kumar 2015; Choudhary et al. 2016; Zhang and Sonnewald 2017).

The net outcome of the host-pathogen interaction under drought conditions, is dependent on plant genotype, the nature of the pathogens and the timing, severity and duration of the stress (Asselbergh et al. 2008; Sinha et al. 2016; Dossa et al. 2017; Songy et al. 2019). Concurrent drought stress most often aggravates disease (Mayek-Pérez et al. 2002; Vemanna et al. 2019), yet it can also trigger resistance (Achuo et al. 2006; Ramegowda et al. 2013). In grapevine, a lot of research has been done on drought stress, but less is known about pathogen stress and there is almost no knowledge about simultaneous occurrence of drought and pathogen stress (Choi et al. 2013). A few studies provide information about subsequent, rather than simultaneous, drought and pathogen stress, after inoculation with *Plasmopara viticola* on intact plants (Roatti et al. 2013) or with *Botrytis cinerea* on detached leaves (Hatmi et al. 2015).

The aim of the current study was to gain more insight into the mechanisms underlying drought and pathogen stress, both individually and simultaneously, and the influence of their interplay on disease susceptibility. Fully irrigated and drought-stressed vines were artificially inoculated with *P. viticola.* This obligate biotroph causes grapevine downy mildew, one of the most important diseases in European viticulture (Eurostat 2000; Pertot et al. 2017). An interaction between both stresses is to be expected, since the stomata, the site of entry of *P. viticola*, are the plants' first line of defense against drought stress. *P. viticola* is able to manipulate stomatal behavior (Allègre et al. 2007), facilitating the infection and thus potentially altering the drought response. The experiment was conducted using an introduced cultivar (Chardonnay) and a drought-tolerant, indigenous cultivar (Xynisteri) in the natural, hot and dry climate of Cyprus.

Materials And Methods

Site description and plant material

The research was conducted on a sun exposed area in Limassol, Cyprus (34°42'N, 32°59'E; elevation: 100 m a.s.l.), during 22 rainless days in May 2018. The climate is Mediterranean, with hot and dry summers. Supplementary Fig. S1 presents the climatic data, recorded with an on-site data logger (Kistock KH 250; Kimo). On an average day during the experiment, a maximum temperature of 38.3 °C was achieved in full shade, corresponding with 24% RH. On an average night, the minimum temperature dropped to 19.5 °C and the relative humidity reached 70%.

This study comprised two cultivars of *Vitis vinifera*, Xynisteri and Chardonnay. Xynisteri is the main white grape cultivar grown in Cyprus, while Chardonnay, one of the most planted white grape cultivars internationally, has been introduced in Cyprus. In 2014, they respectively covered 30.2% and 1.6% of the ca. 6,142 ha viticultural area of Cyprus (Statistical service of Republic of Cyprus 2016). Of each cultivar, 60 self-rooted cuttings were planted in 5-liter polyethylene pots containing soil, originating from the traditional vineyard area in Limassol. The soil properties were previously described by Tzortzakis et al. (2020) and briefly, the soil had a clay-loam texture, organic matter of 2.19%; total $CaCO_3$ of 66.9%; pH of 7.42; electrical conductivity (EC) 0.28 of mS cm^{-1}. The plants were grown in field conditions and were automatically irrigated at field capacity using a drip irrigation system. Three months after planting, the plants were uniformly distributed over 12 treatment groups. For each treatment, five replicates were used per cultivar. The experimental set-up is shown in Fig. 1. Each group was treated with one of four abiotic stress treatments (7 or 14 days of full or deficit irrigation) to assess the effect of short and prolonged drought stress. Two groups were sampled destructively at 7 and 14 days of treatment (dot). *In vitro* inoculations were performed on disks of these leaves. In the evening, the remaining intact plants were inoculated with either pathogen or water. For these plants, the irrigation regime was maintained until disease evaluation. Some leaves were sampled at 9 and 16 dot to establish the effect of pathogen attack at 1.5 days post inoculation (dpi) on drought-stressed plants.

Abiotic stress

Plants were either well-watered in the full irrigation control treatment or exposed to drought stress by deficit irrigation. Fully irrigated plants received irrigation at field capacity from an automatic drip system, every 6 hours for 5 min. Deficit irrigation was maintained at 40% of the full irrigation, based on the volumetric water content of the soil (VWC). The deficit-irrigated plants were irrigated manually every two days. To verify and accurately adjust the irrigation, the VWC was measured daily in 8 randomly chosen pots using a portable Time-Domain Reflectometer (TDR) (FieldScout TDR 300 Soil Moisture Probe; Spectrum Technologies) with 4.7 inch rods (Supplementary Fig. S2).

Biotic stress

To examine the combined effect of abiotic and biotic stress on the vine, a pathogen stress was imposed on the intact plants after 7 or 14 days of drought stress. *Plasmopara viticola* isolate Fcpv1, obtained from Chardonnay in France, was grown for 10 days at 22 °C on detached Chardonnay leaves on water agar

(0.65%). Sporangia were collected with distilled water and the suspension was adjusted to 2.5×10^4 sporangia mL^{-1}. The artificial inoculation was performed in the evening. The abaxial sides of all leaves were sprayed until run-off with 3 mL sporangia suspension of *P. viticola*. The control plants were sprayed with distilled water. The irrigation regimes were maintained until the disease evaluation. Since *P. viticola* needs 95–100% relative humidity during the night for an optimal infection and sporulation, each plant was equipped with a container of water and a humid plastic cover in the evening. To prevent extreme temperature development within the cover, the cover was removed in the morning and a light shade was created using a shadow mesh.

Sampling and disease evaluation took place at 1.5 and 7 dpi respectively. Each plant was evaluated according to the following classes: 0, no symptoms; 1, few oil spots with little to no sporulation; 2, moderate symptoms and non-spreading sporulation; 3, clearly diseased with spreading sporulation; 4, severe symptoms with dense sporangiophore carpets.

Field measurements

At 3, 7, 9, 14 and 16 dot, stomatal conductance, chlorophyll fluorescence and chlorophyll content were recorded. The measurements were conducted on the 4th or 5th leaf starting from the apical meristem on randomly chosen plants at mid-morning, 4 h after onset of light. The stomatal conductance to water vapor (g_s) was measured on three to five plants, using a transient state diffusion porometer (AP4; Delta-T Devices). The chlorophyll fluorescence (F_v/F_m), an indicator of the maximum quantum efficiency of Photosystem II, was monitored on three or four plants after exposure to darkness for 20 minutes with a dark adaptation pin using a chlorophyll fluorometer (OS30p; Opti-Sciences). The chlorophyll content per unit leaf area was estimated using a non-destructive Soil Plant Analysis Development (SPAD) meter (SPAD 502 Plus; Spectrum Technologies). SPAD measurements were conducted twice on the same leaf of five or six plants.

In vitro **assessment of disease susceptibility**

The 3rd and 4th leaf, counted from the apex, sampled at 7 and 14 dot before application of biotic stress treatment, were used to investigate the effect of the previous exposure to drought stress on the susceptibility to *P. viticola*. Leaf disks (11 mm diameter) were treated with 20 µL distilled water or 20 µL *P. viticola* sporangia suspension containing 2.5×10^4 sporangia mL^{-1} and incubated on water agar (0.65%) at 22 °C. At 5 dpi, the number of sporangiophores was counted to assign each disk to one of the following classes: 0, 0 sporangiophores; 1, 1–6 sporangiophores; 2, 7–20 sporangiophores; 3, > 20 sporangiophores; 4, dense sporangiophore carpet. An average of 60 disks were evaluated per treatment.

Quantification of phytohormones

The levels of abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA) and salicylic acid (SA) were determined in leaves sampled at 7, 9, 14 and 16 dot. For each of the five replicates per treatment, two leaves were pooled, immediately frozen in liquid N_2 and kept at -80 °C until analysis. The procedure

for quantification of phytohormones is described in detail by Haeck et al. (2018). The ground tissue (100 mg) was incubated with 5 mL modified Bieleski extraction solvent (methanol/water/formic acid 75:20:5, v/v/v) for 20–24 h at -80 °C. After this cold extraction, filtration (30 kDa Amicon® Ultra centrifugal filter unit, Merck Millipore, Overijse, Belgium) and evaporation (TurboVap® LV, Biotage, Uppsala, Sweden), the extracts were reconstituted in 0.5 mL methanol/water/formic acid (20:80:0.1, v/v/v). Chromatographic separation was performed on an ultra-high-performance liquid chromatography system (U-HPLC, Thermo Fisher Scientific) equipped with a Nucleodur C18 column (50 × 2 mm; 1.8 μm particle diameter). Mass spectrometric analysis was achieved in targeted single ion monitoring mode on a Q-Exactive™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with a heated electrospray ionization source, at a resolution of 70,000 full width at half maximum. In negative ionization mode, SA, ABA and JA were measured using an elution gradient (300 μL min^{-1}) of (A) methanol and (B) water, both with 0.01% formic acid. The formic acid concentration of solvent B was adjusted to 0.1% for measurement of IAA in positive ionization mode. The following linear gradient was applied (solvent A): 0–1 min at 20%, 1-2.5 min from 20 to 45%; 2.5-9 min from 45 to 100%; 9–10 min at 100%; 10–14 min at 20%. External and deuterated internal (d_4-SA at 200 μg L^{-1}, d_6-ABA and d_5-IAA at 1 μg L^{-1}) standards were used for accurate quantification of the hormone content.

Quantification of photosynthetic pigments

Leaf samples were collected at 7, 9, 14 and 16 dot with five replications per treatment, each consisting of a pool of two leaves. Leaf tissue (100 mg) was incubated in a heat bath at 65 °C for 30 min, with 10 mL dimethyl sulfoxide (DMSO). The absorbance of the extract was measured at 645 nm and 663 nm, using a microplate spectrophotometer (Thermo Scientific, Multiskan GO) and chlorophyll a (Chl a) and chlorophyll b (Chl b) concentrations were calculated as described by Richardson et al. (2002).

Quantification of hydrogen peroxide content, lipid peroxidation and proline

For the quantification of hydrogen peroxide (H_2O_2), lipid peroxidation, in terms of malondialdehyde (MDA) content, and proline, two leaves were sampled and pooled for each of the five plants per treatment at 7, 9, 14 and 16 dot. Fresh leaves were immediately frozen in liquid N_2 and kept at -80 °C until analysis. Before analysis, ground leaf tissue (200 mg) was homogenized with ice-cold 0.1% trichloroacetic acid (TCA). The extract was centrifuged and the supernatant was used for the quantification of H_2O_2 and MDA (Chrysargyris et al. 2017). For the quantification of H_2O_2, 0.5 mL of the supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (PPB) (pH 7.0) and 1 mL of 1M potassium iodide (KI). The content of H_2O_2 was calculated using standards of 5 to 500 μM of H_2O_2 and a calibration curve was plotted accordingly. The absorbance was measured at 390 nm. For the MDA content, 0.5 mL of the supernatant was incubated with 1.5 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA at 95 °C for 25 min. The reaction was stopped in an ice bath and the absorbance was measured at 532 nm and 600 nm. The MDA content was calculated using the extinction coefficient of 155 mM cm^{-1}.

Proline content was also determined using this frozen ground tissue. Leaf tissue (200 mg) was homogenized in 2 mL of 3% aqueous sulfosalicylic acid (SSA). Extracts were then centrifuged and 1 mL of the supernatant was incubated with 1 mL of acid ninhydrin and 1 mL of glacial acetic acid, for 1 h at 100 °C. Then, the formed chromogen was extracted with toluene and the absorbance was measured at 520 nm, using toluene as blank. The proline concentration was determined using serial dilutions (0-100 µg mL^{-1}) of D-proline (Khedr 2003).

Quantification of antioxidant enzymes

The ground leaf samples were also used for the determination of the activity of the antioxidant enzymes. The tissue (200 mg) was homogenized with 3 mL ice cold 50 mM potassium phosphate buffer (pH 7.0), including 1 mM ethylenediaminetetraacetic acid (EDTA), 1% w/v polyvinylpolypyrrolidone (PVPP), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% polyethylene glycol tert-octylphenyl ether (Triton X-100). The homogenate was centrifuged at 16,000 g for 20 min, at 4 °C. The supernatant was collected and an aliquot was first used to determine the protein content via the Bradford method (1976), with bovine serum albumin (BSA) as the protein standard.

Catalase (CAT, EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm for 3 min, as assayed by Jiang and Zhang (2002). The reaction mixture contained 100 mM PPB (pH 7.0), plant extract and 200 µL of 75 mM of H_2O_2. Results were expressed as CAT units per milligram of protein. One unit of enzyme decomposed 1 µmol of H_2O_2 per min.

Superoxide dismutase (SOD, EC 1.15.1.1) was assayed using the photochemical method. The reaction mixture (1.5 mL) contained 50 mM PPB (pH 7.5), 13 mM methionine, 75 µM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 2 µM riboflavin and an enzyme aliquot. Reaction started after the addition of riboflavin. Tubes containing the reaction were then placed under a light source of two 15-watt fluorescent lamps for 15 min. The reaction was stopped by placing the tubes in the dark. Reaction without the extract developed maximal color (control) and non-irradiated mixture was used as a blank. The absorbance was determined at 560 nm and activity was expressed as SOD units per mg of protein. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate (Chrysargyris et al. 2018).

Peroxidase activity (POD, EC 1.11.1.7) was determined according to the method used by Tarchoune et al. (2012). POD activity was assayed using pyrogallol, following the increase in absorbance at 430 nm, after the oxidation to purpurgallin. The reaction mixture of 2 mL contained 1,665 µL of 100 mM PPB (pH 6.5), 200 µL of 100 mM pyrogallol and 50 µL of extract. The reaction started with the addition of 85 µL of 40 mM H_2O_2. The increase in absorbance at 430 nm was measured on a kinetic cycle for 3 min.

Calculations were performed using the coefficient of extinction of 2.47 mM cm^{-1}. One POD unit was defined as the amount of enzyme needed to decompose 1 µmol of H_2O_2 per min.

Statistical analysis

All statistical analysis were conducted using R, version 3.6.1 (R Core Team 2019). Normality and homoscedasticity were verified with the Shapiro-Wilk's and Levene's test (p = 0.05). Data meeting the assumptions, were treated with one-way analysis of variance (ANOVA), followed by Tukey's HSD (honestly significant difference) to compare the means (p = 0.05). Nonparametric data were analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney U-test (p = 0.05). For the analysis of the interactions between the cultivar, drought and pathogen stress, linear regression analysis was performed. A generalized least squares (GLS) model was improved by eliminating interaction terms until the lowest Akaike Information Criterion score (AIC) was reached.

Results

Basal differences between cultivars

To assess the basal differences between Xynisteri and Chardonnay, only plants without abiotic or biotic stress were taken into account (Fig. 2). Strikingly, in Chardonnay, ABA and SA levels were more than double and chlorophyll levels almost double the levels of Xynisteri. H_2O_2 content tended to be slightly elevated in Chardonnay. In contrast, POD and SOD activities and IAA and proline content were significantly higher in Xynisteri than in Chardonnay and MDA content tended to be slightly higher in Xynisteri. For CAT activity and JA content, no significant differences were observed between the cultivars.

Effect of drought stress on disease susceptibility

To assess the effect of the previous exposure to drought stress on the susceptibility to *P. viticola*, plants were first exposed to deficit irrigation, either for 7 or 14 days, before *in vitro* inoculation of leaf disks with the pathogen (Fig. 3a). When watered sufficiently (full irrigation), Xynisteri showed a significantly higher disease severity than Chardonnay. On disks from non-stressed Chardonnay plants, almost no sporulation was present. However, the Chardonnay leaf disks became more susceptible to *P. viticola* when exposed to 7 days of deficit irrigation. On Xynisteri leaf disks, *P. viticola* was able to develop fast, irrespective of this exposure to drought stress. As the duration of the previous drought exposure increased from 7 to 14 days, the disease severity increased in both cultivars. Especially in Chardonnay, the long exposure to drought stress drastically enhanced susceptibility, reaching a level similar to Xynisteri.

To assess the combined effect of the abiotic and biotic stress on the plant, disease was evaluated on plants subjected either to 7 or 14 days of irrigation treatment before being sprayed with a *P. viticola* sporangia suspension or distilled water (Fig. 3b). The irrigation regime was maintained until the moment of disease evaluation, at 7 dpi. Interestingly, in fully irrigated conditions, the indigenous cultivar Xynisteri showed more severe symptoms than the introduced cultivar Chardonnay. While the fully irrigated plants showed clear disease symptoms, the symptoms on drought-stressed plants at 7 dot remained almost completely absent. Comparing deficit-irrigated plants inoculated at 7 and 14 dot, the resistance to *P. viticola* did not significantly change with longer exposure to deficit irrigation before inoculation.

Field measurements

Single drought stress

Figure 4 shows the changes in the physiological parameters due to the irrigation and inoculation. The statistical analysis of the single stress treatments is indicated in Supplementary Table S1. The temporal changes in physiological parameters during drought stress are presented in Supplementary Fig. S3. Drought stress had a profound effect on the leaf stomatal conductance. After 3 days of deficit irrigation, the stomatal conductance had already plummeted in both cultivars (Supplementary Fig. S3). Drought stress (Fig. 4, ' vs ') did not affect the SPAD values, which are a measure for the chlorophyll content per unit leaf area, initially (9 dot), though a slight reduction was observed during prolonged drought stress (16 dot) for Chardonnay. Chlorophyll fluorescence increased significantly in Xynisteri, but not in Chardonnay, after 3 and 7 days of deficit irrigation (Supplementary Fig. S3). After 14 days of prolonged stress, the drought caused the chlorophyll fluorescence to decrease sharply in both cultivars. The higher loss of chlorophyll fluorescence at 16 dot in Chardonnay indicates this cultivar suffered more than Xynisteri (Fig. 4).

Single pathogen stress

No significant differences in physiological parameters were observed between plants inoculated with water and plants inoculated with *P. viticola* for the same irrigation regime (Supplementary Table S1). However, an increasing trend in stomatal conductance due to the pathogen infection was observed at 16 dot (Fig. 4, ' vs '). SPAD was slightly lower in pathogen-inoculated compared to water-inoculated plants at 1.5 dpi.

Combined stress

When both stresses were combined (Fig. 4, '), the net effect on the physiological parameters was comparable to the strong effect of drought stress. The physiological parameters between drought-stressed plants with and without pathogen were not statistically different (not shown). Xynisteri demonstrated a slightly higher stomatal conductance under combined stress compared to plants under single drought stress. Conversely, a slightly lower stomatal conductance was found in Chardonnay under combined drought stress. Furthermore, chlorophyll fluorescence seemed to decrease in Xynisteri under combined stress compared to the single drought stress, but not in Chardonnay.

Phytohormone balance

Single drought stress

Figure 5 shows how drought and pathogen stress affect the phytohormone content. The statistical analysis of the single stress treatments is indicated in Supplementary Table S1. The interactions between cultivar, pathogen stress and irrigation and its duration were analyzed with a regression model (Supplementary Tables S2-S3). A short (9 dot) or prolonged (16 dot) deficit irrigation had a profound effect on the phytohormone balance (Fig. 5, ' vs '). Both cultivars reacted to the drought stress by

significantly increasing ABA and decreasing JA, resulting in similar levels in both cultivars. Considering the large basal differences in ABA, Xynisteri was producing much more ABA than Chardonnay in response to drought. Indeed, the ABA response to the drought stress was likely to be cultivar-dependent (Supplementary Table S2). Moreover, ABA levels seemed to increase when the drought stress prolonged. Drought stress was also related to a significant increase in IAA in both cultivars, which seemed to be higher as the drought stress prolonged. Finally, the SA response to drought stress was significantly dependent on the cultivar (Supplementary Table S2). Chardonnay responded to drought stress by dropping its SA content, especially at 16 dot (Supplementary Table S1), while still maintaining levels higher than Xynisteri. No clear SA response to drought was observed for Xynisteri (Fig. 5).

Single pathogen stress

To assess the hormonal changes in the cultivars upon pathogen infection, plants were sprayed with water or *P. viticola* inoculum. Analysis was performed on samples taken at 1.5 dpi from fully irrigated plants (Fig. 5, ' vs '). Independent repetitions occurred at 9 and 16 dot, with only a slight change in plant age. Although Xynisteri was more susceptible to *P. viticola* than Chardonnay, the hormonal responses of the cultivars to the pathogen were similar. Compared to the water-sprayed plants, pathogen-inoculated plants accumulated more JA and IAA (Fig. 5). In the linear regression, there are indications that IAA is positively affected by the interaction of the pathogen, at least on Xynisteri (Table S3). The SA levels seemed slightly elevated and the level of ABA remained unaffected by the pathogen (Fig. 5).

Combined stress

The cultivars were subjected to 7 or 14 days of deficit irrigation, before being sprayed with water or *P. viticola* inoculum, to determine the combined effect of abiotic and biotic stress. The irrigation regime was maintained and samples for analysis were taken 1.5 dpi at 9 or 16 dot (Fig. 5, ').

Interestingly, ABA levels, already strongly increased due to drought, rose even more 1.5 days post inoculation with the pathogen. The interaction between pathogen and drought stress was highly significant for ABA (Supplementary Table S2). The additional ABA accumulation indicated that drought-stressed plants responded to the pathogen, although no symptoms were observed under drought stress (Fig. 3b). Similarly, an increasing trend in IAA was observed in drought-stressed Xynisteri, already demonstrating higher levels of IAA due to the drought. In drought-stressed Chardonnay, the presence of the pathogen did not cause an additional rise in IAA. A significant interaction between drought and pathogen stress was also observed for JA (Supplementary Table S2). The accumulation of JA in response to the pathogen in fully irrigated plants, disappeared when deficit irrigation was applied (Fig. 5). A similar trend was observed for SA.

Chlorophylls and oxidative balance

Single drought stress

Figure 6 demonstrates the impact of drought and pathogen stress on chlorophyll and oxidative parameters. The statistical analysis of the single stress treatments is indicated in Supplementary Table S1. The interactions between cultivar, pathogen stress and irrigation and its duration were analyzed with a regression model (Supplementary Tables S2-S3). To assess how a short and prolonged drought stress affect the cultivars, plants were subjected to full or deficit irrigation for 9 or 16 days, before samples were collected for analysis of the chlorophyll content and the oxidative parameters (Fig. 6, ' vs '). Even when drought-stressed, the basally higher activities of SOD and POD in Xynisteri were maintained, just as the chlorophyll levels in Chardonnay remained significantly higher than in Xynisteri. At first, POD activity increased and MDA decreased in Xynisteri in response to drought stress (9 dot), while the chlorophyll, SOD and CAT activity tended to be elevated. However, when the drought stress persisted (16 dot), POD and SOD activity seemed to diminish, and H_2O_2 levels to rise. Xynisteri's response did no longer include changes in chlorophyll, MDA or CAT.

This response of Xynisteri to 16 days of deficit irrigation, was comparable to the earlier response of Chardonnay to 9 days of irrigation. The only difference in drought response between Chardonnay at 9 dot and Xynisteri at 16 dot, was the trend for CAT. While CAT activity dropped significantly in Chardonnay at 9 dot (Supplementary Table S1), it remained unchanged in Xynisteri at 16 dot. Continued drought stress (16 dot) eventually caused a significant increase in SOD activity in Chardonnay (Supplementary Table S1). Its chlorophyll content seemed to lower and the MDA content and POD and CAT activity seemed to increase.

Single pathogen stress

To determine how cultivars responded to *P. viticola*, plants were sprayed with water or pathogen inoculum. Samples for analysis were taken from fully irrigated plants 1.5 dpi (Fig. 6, ' vs '). In this case, both time points (9 or 16 dot) could be seen as a repetition, with only a small change in plant age. Despite their difference in disease severity (Fig. 3b), the responses of the cultivars to the pathogen were similar concerning the parameters tested. The CAT activities were lowered significantly and a decrease was observed for SOD and POD. What stood out most, was the significant burst in proline associated with the pathogen-inoculated plants (Supplementary Table S1), especially at 16 dot. At 16 dot, MDA rose and chlorophyll decreased slightly, SOD activity was significantly reduced in Xynisteri and H_2O_2 content seemed to go up in Chardonnay.

Combined stress

To examine the combined effect of both abiotic and biotic stress, the plants were subjected to 7 or 14 days of deficit irrigation, before being sprayed with water or *P. viticola* inoculum. The irrigation regime was maintained and samples for analysis were taken 1.5 dpi at 9 or 16 dot (Fig. 6, '). Mostly, the responses of deficit and fully irrigated plants to the pathogen were similar. As the drought prolonged (16 dot) in Chardonnay however, chlorophyll content seemed to increase with pathogen inoculation, while it seemed to decrease in full irrigation. Similarly, in Xynisteri, the combination of prolonged drought and pathogen stress seemed to buffer Xynisteri against loss of chlorophyll levels by pathogen stress (Fig. 6). Indeed, for chlorophyll, the interaction between abiotic stress, its duration and biotic stress is significant

(Supplementary Table S2). Thus, the chlorophyll loss at 1.5 dpi in Xynisteri might be associated with the successful infection by *P. viticola*. The MDA response to the pathogen depended primarily on the interaction with cultivar, drought stress and its duration (Supplementary Table S2). MDA too, seemed rather associated with successful infection: MDA increased in fully irrigated plants at 16 dot when inoculated with the pathogen. Deficit-irrigated, inoculated plants at 16 dot exhibited levels of MDA similar to the non-inoculated plants (Fig. 6).

CAT and POD activities were significantly reduced in pathogen-inoculated plants under full irrigation. Although no disease symptoms were seen on the drought-stressed plants (Fig. 3b), a similar reduction was observed in plants subjected to drought and pathogen stress. The clear rise in proline in pathogen-inoculated plants was present in both irrigation regimes, indicating proline was also associated with the inoculation, rather than the disease incidence.

Principal component analysis

Figure 7 shows the principal component analysis (PCA). Although the cultivars and irrigation and pathogen treatment were used as supplementary variables, not taking part in the construction of the dimensions, they allow grouping of the data. A strong correlation was found between the first dimension (Dim1) and the cultivar (Table 1). Indeed, there is a good horizontal separation between both cultivars along the axis of the first dimension (Fig. 7a). The association with the first dimension indicates that the differences between the cultivars, both basally and in their response to the stresses, explained most of the variation in our dataset. Chardonnay was mostly associated with higher values for chlorophyll, SA, ABA and H_2O_2, while Xynisteri was associated with higher POD, SOD, CAT and MDA values (Table 2). The second dimension (Dim2) was mostly correlated to the irrigation regime and, to a lesser extent, the duration of this treatment. In the PCA, a horizontal shift is visible according to drought stress and its duration (Fig. 7b). Drought-stressed plants were mostly associated with ABA, CAT, IAA, but also with chlorophylls, H_2O_2, SOD and POD. Fully irrigated plants were mainly correlated with JA, and SA to a lesser extent (Table 2). Finally, the third dimension was primarily correlated to the pathogen stress. In Fig. 7c, water (mock) and pathogen-inoculated plants separate clearly along the vertical axis (Dim3), irrespective of the disease severity. Proline, IAA and MDA seem to be strongly correlated and are associated with pathogen-inoculated plants.

Table 1

Significant square correlation ratios (R²) indicating the correlation between the dimensions of the PCA and the supplementary variables cultivar (Chardonnay or Xynisteri), irrigation (full or deficit), duration (of the abiotic stress; 9 or 16 dot) and pathogen (*P. viticola* or water inoculation), with corresponding p-values. The supplementary variables were not involved in the construction of the dimensions

Supplementary variables	R^2			P-value		
	Dim1	Dim2	Dim3	Dim1	Dim2	Dim3
Cultivar	0.703			3.12E-22		
Irrigation		0.355			5.40E-09	
Duration		0.188	0.063		5.74E-05	2.51E-02
Pathogen		0.089	0.490		7.12E-03	5.18E-13

Table 2

Significant correlation coefficients of the active variables, describing the construction of the different dimensions of the PCA, with corresponding p-values. The colors indicate a positive (blue) or negative (red) correlation

Active variables	Correlation			P-value		
	Dim1	Dim2	Dim3	Dim1	Dim2	Dim3
ABA	-0.292	0.624		8.61E-03	6.21E-10	
IAA		0.361	0.602		1.01E-03	3.51E-09
JA		-0.806			1.90E-19	
SA	-0.523	-0.283		6.50E-07	1.11E-02	
H_2O_2		0.369			7.67E-04	
CAT	0.505	0.586		1.75E-06	1.14E-08	
POD	0.850	0.225		1.88E-23	4.48E-02	
SOD	0.815	0.382		3.86E-20	4.69E-04	
MDA	0.481		0.467	6.24E-06		1.25E-05
Proline			0.812			5.85E-20
Chl a	-0.759	0.428		3.32E-16	7.37E-05	
Chl b	-0.793	0.413		1.84E-18	1.41E-04	

Along the horizontal axis (Dim2) of Fig. 7c, plants are separated according to the disease severity, symptomless plants on the right and the most diseased plants on the left. When clustering according to drought stress in the same dimensions (Fig. 7b), the group showing no symptoms overlap with the

drought-stressed group. Resistance is primarily observed in the 1st quadrant, since the lion share of the plants in the 3rd and 4th quadrant was not inoculated with the pathogen. The diseased plants are mainly clustered in the 2nd quadrant. The PCA suggests JA is associated with the infection under full irrigation. Interestingly, this PCA indicates that ABA and IAA play a role in the drought-induced resistance, in which JA is no longer involved.

Discussion

Phytohormones

Single drought stress

The single drought stress severely affected the phytohormone balance of the drought-tolerant cultivar Xynisteri and the drought-sensitive cultivar Chardonnay, respectively native and introduced in the investigated climate. In these conditions, ABA, generally considered the key hormone underpinning mechanisms that regulate drought stress responses in plants, appeared to govern the complex hormone crosstalk by antagonizing JA and SA. For both cultivars, drought stress increasingly triggered ABA as the duration extended, but negatively impacted JA. SA was lowered too, but primarily in the drought-sensitive Chardonnay. Although most studies observe that JA and SA are involved in drought stress responses in addition to ABA, (Tiwari et al. 2017), the negative interaction of ABA with JA and SA has also been reported before (Sánchez-Vallet et al. 2012; Hussain et al. 2019). Multiple nodes allow interference of ABA with the JA-ethylene pathway (Anderson et al. 2004), but whether their interaction is antagonistic (Anderson et al. 2004) or synergistic (Adie et al. 2007) is strongly depend on the conditions. The suppressive effect of ABA on the SA signaling pathway (Mohr and Cahill 2003; Mauch-Mani and Mauch 2005; Yasuda et al. 2008), has been shown for grapevine in particular by Wang et al. (2018), where elicitation with exogenous ABA led to a gradual reduction of SA.

Our data demonstrates that drought stress also caused the levels of IAA to increase in both cultivars. The basally higher IAA in Xynisteri might contribute to its drought tolerance. Although not as thoroughly studied in this context as ABA, endogenous IAA levels have been reported to increase during the grapevine defense response against drought (Haider et al. 2017). Through its crosstalk with reactive oxygen species (ROS), IAA can help plants to adjust their growth to unfavorable conditions (Tognetti et al. 2012). Previous studies have associated elevated auxin with induction of abiotic stress-related genes, activation of the antioxidant response and reduction in ROS accumulation (Kim et al. 2013; Cheol Park et al. 2013; Shi et al. 2014; Bielach et al. 2017).

Single pathogen stress

P. viticola was able to infect irrigated vines easily in the extreme weather conditions of Cyprus, with high light intensities and maximum daily temperatures reaching 45 °C in the shade, although previous studies have shown both high temperature (Caffi et al. 2016; Angelotti et al. 2017) and light intensities (Williams et al. 2007) inhibit sporulation. The nights, with minimum temperatures between 15 and 25 °C and

relative humidity reaching 80 to 90%, were optimal for infection. Both *in vitro* and *in planta* inoculations demonstrated that Xynisteri was more susceptible to *P. viticola* than Chardonnay when irrigated. Remarkably, our results for fully irrigated plants indicate the infection by the pathogen was also associated with elevated IAA. IAA appeared to be mainly correlated with proline, which accumulated in both cultivars. The higher basal IAA and proline levels in Xynisteri could be related to its higher disease susceptibility. It is well-known that some pathogens are able to upregulate the plants auxin signaling, in order to suppress plant defenses, while others are able to synthesize IAA themselves through various pathways, to increase their pathogenesis (Yin et al. 2014). IAA levels during *P. viticola* infection have not been studied before, so the question of the origin and function of the IAA accumulation remains. The accumulation of proline associated with elevated IAA has been observed in IAA treated plants (Joshi et al. 2011). Like auxin, proline is also involved in numerous developmental processes (Trovato et al. 2018), which can help maintain sustainable growth under long-term stress. However, because of its positive correlation with pathogen-triggered IAA, the role of proline in the plant-pathogen interaction is ambiguous. As a ROS scavenger, proline might have been produced as part of the host defense mechanism against the oxidative stress caused in response to the pathogen. However, proline might benefit the pathogen in a similar way, by detoxifying ROS, which restrict pathogen development.

Contrary to the abiotic stress, we found pathogen stress acted positively on JA and SA contents of both cultivars, without an apparent effect on ABA levels. The upsurge of JA and SA at 1.5 dpi in infected, fully irrigated plants demonstrates the plants were activating their defense mechanism. SA is basally higher in Chardonnay and might be part of its more successful defense against *P. viticola*. The roles of JA and SA have been extensively studied in resistant cultivars, where both phytohormones strongly accumulate after infection with *P. viticola* (Guerreiro et al. 2016). SA as well as JA-mediated defense responses are implicated in the resistance to *P. viticola* (Polesani et al. 2010; Marchive et al. 2013; Gauthier et al. 2014; Figueiredo et al. 2015; Li et al. 2015). Moreover, exogenous JA has been shown to protect grapevine leaf disks against *P. viticola* through callose deposition (Hamiduzzaman et al. 2005). The dynamics of endogenous phytohormones during compatible interactions with *P. viticola* have however not been explicitly investigated. Polesani et al. (2010) and Li et al. (2015) did observe increases in JA, coupled to very strong rises in methyl jasmonate (MeJA), in successful infections. Both endogenous levels increased from 12 to 48 hpi (hours post inoculation), but perished once the tissue was completely invaded (Polesani et al. 2010), implicating the involvement of JA and MeJA defenses in the early developmental stages of the pathogen in compatible interactions.

Combined stress

Combined abiotic and biotic stress is a different story entirely. When the drought and pathogen stress occurred simultaneously, the two stress responses interacted. Remarkably, under continued deficit irrigation, disease symptoms were no longer observed in Chardonnay or Xynisteri. Even more interesting is our finding that JA and SA contents were low and no longer substantially contributed to the pathogen defense response. Our results reveal that, under concurrent stress, ABA dominated the responses to the pathogen stress occurring under full irrigation, antagonizing JA and SA. Furthermore, a significant,

additional rise in ABA was observed in inoculated compared to non-inoculated drought-stressed plants, although under full irrigation, the infection did not trigger ABA. Thus, we hypothesize that ABA, rather than JA or SA, is involved in the observed drought-induced resistance to *P. viticola*. But how can ABA contribute to hindering the infection of *P. viticola*?

Considered a global regulator of plant stress responses, ABA is crucial in the response of plants to multiple stresses (Atkinson and Urwin 2012). Its role in pathogen defense is poorly understood. Whether ABA acts as a positive or negative regulator of disease resistance, is dependent on the stage of infection and pathosystem, yet seems to be unrelated to the pathogen life style or mode of attack (Asselbergh et al. 2008). Although most studies have established an antagonistic relationship between ABA and disease resistance (Audenaert et al. 2002; Mohr and Cahill 2003; Anderson et al. 2004; Asselbergh et al. 2007; Gupta et al. 2017), treatment of detached grapevine leaves with exogenous ABA has been shown to result in a reduction of *P. viticola* infection, albeit only in high concentrations (Hamiduzzaman et al. 2005; Allègre et al. 2009).

ABA can be involved in pre-invasive defense, preventing pathogen penetration by controlling rapid stomatal movement (Melotto et al. 2006). Our data suggests however that the pathogen was not stopped during the pre-invasive defense. Despite their differences in disease susceptibility, both fully and deficit-irrigated, pathogen-inoculated plants still showed major changes in IAA, proline, CAT, POD and to a lesser extent SOD. Their independence from the irrigation treatment at this infection stage (1.5 dpi), punctuates the infection in the deficit-irrigated plants ceased post-penetration. It indicates *P. viticola* was able to penetrate the substomatal cavities, even though the stomatal conductance was markedly reduced in response to the deficit irrigation. Notably, an additional rise in ABA was observed in deficit-irrigated plants after inoculation with the pathogen. This additional rise in ABA could be key to the post-invasive resistance to this pathogen. During the post-invasion defense, ABA is implicated in callose (Ton and Mauch-Mani 2004; Adie et al. 2007) and stilbene (Wang et al. 2018) accumulation, thus limiting the pathogen spread. ABA has also been found to accumulate strongly in some genetically resistant *Vitis* species after *P. viticola* inoculation (Liu et al. 2016; Wang et al. 2018). In many resistant *Vitis* species, most infections never advance beyond the assessed developmental stage (24–48 hpi, Unger et al. 2007; Polesani et al. 2010).

However, while continued exposure to drought induced resistance, we discovered that detached leaves from drought-stressed plants became more susceptible to this pathogen when inoculated in humid, temperate conditions. This indicates the drought-induced, ABA-mediated resistance depends on a rapid defense response, which can be inverted in a very short time. The fast turnover of the drought-induced resistance could explain why Roatti et al. (2013) did not report a reduction in disease severity when *P. viticola* was inoculated at the end of a deficit irrigation period. Because of the striking difference in disease severity during and after exposure to deficit irrigation, it is unlikely a physical barrier is the source of the ABA-mediated, post-invasive resistance. Whatever it may be, the response is strongly dependent on the ABA concentration, which is determined by ABA production, transport and catabolism. The rate at which stress ABA is catabolized, might be proportional to the amount of stress ABA accumulated (Ren et

al. 2007). Hence, once the drought stress is lifted and stress ABA is no longer synthesized, the high levels of ABA cannot be sustained. We hypothesize that the recovered disease susceptibility in detached leaves of drought-stressed plants is linked to their inability to maintain sufficiently high ABA levels and to timely restore the adverse effects of drought on its pathogen defense. After all, the drought severely interfered with the pathogen response, including induced IAA and antioxidant enzyme activity and antagonized JA and SA levels. From this point of view, it is not surprising that post drought, Chardonnay partially lost its higher tolerance to the pathogen. Its *in vitro* susceptibility even increased with the duration of the previous drought stress. Apart from the increased adverse effects, additional ABA accumulated when the deficit irrigation prolonged. This potentially caused lower ABA levels post drought, as a result of the increased ABA catabolism. The previous irrigation regime also deteriorated Xynisteri's pathogen defense, but this cultivar was already extremely susceptible under full irrigation.

The changing climate and the practices used to mitigate its effects, have a profound impact on plant pathogens. Based on these results, irrigation might render pathogens to become a sudden threat to the agroecosystem sustainability. Full irrigation of a drought-tolerant cultivar enhanced its susceptibility to downy mildew infection. The drought-tolerant cultivar can easily be grown with no or ample irrigation, hereby inducing resistance, but for the introduced cultivar, the irrigation is of greater importance. The increasing carbon footprint coupled to the additional irrigation and disease control measures, underline the growing importance of the "right plant for the right place". Moreover, the enhanced disease susceptibility found in the *in vitro* assessment, prompts the question as to whether vines, under the studied field conditions, could become more vulnerable to *P. viticola* during a rain event following a drought period.

Stomatal conductance and photosynthetic parameters

Single drought stress

Since Chardonnay originates from French valleys with humid conditions, this cultivar probably lacks adaptive changes to quickly cope with water stress and might have less sensitive stomatal control than Xynisteri (Schultz 1996; Prieto et al. 2010). The higher basal concentrations of ABA in the leaves of Chardonnay compared to those of Xynisteri, might be prove of its anisohydric behavior (Soar et al. 2006). As a native cultivar in Cyprus, Xynisteri likely has developed fast mechanisms to avoid drought stress. Up to 9 days of deficit irrigation, Xynisteri was even able to increase its chlorophyll fluorescence and content compared to full irrigation. Both cultivars suffered more as the drought stress prolonged. Eventually, the losses in chlorophyll fluorescence, chlorophyll content and SPAD were higher in Chardonnay, indicating that the drought was a greater burden to Chardonnay than Xynisteri.

Single pathogen stress

In plants without drought stress, *P. viticola* was able to infect its hosts proficiently by manipulating them during the infection. Particularly at 16 dot, the pathogen seemed to increase stomatal conductance, potentially as a result of the accumulation of IAA after infection (Pospíšilová 2003), since ABA levels

were not substantially lowered. It is well-known that *P. viticola* is able to manipulate stomatal movements. Stoll et al. (2008) report that stomatal conductance in irrigated plants decreased when infected with *P. viticola*, while other studies observe the pathogen keeping the stomata open by suppressing ABA (Selim 2013) or degrading or blocking its transport (Allègre et al. 2007). Infection also slightly decreased chlorophyll content and SPAD, although chlorophyll fluorescence did not appear to be affected. This biotroph has been shown to lower the photosynthetic rate (Jermini et al. 2010; Figueiredo et al. 2017) through the loss of chlorophyll, the downregulation of chlorophyll a/b binding protein, chlorophyll synthase and Rubisco and the upregulation of chlorophyllase (Gamm et al. 2011). Just 2 days after inoculation, the chlorophyll losses recorded were still small, likely because the chlorophyll content only decreases within the infected lesion (Moriondo et al. 2005; Gamm et al. 2011), and might still have been insufficient to affect the chlorophyll fluorescence (Cséfalvay et al. 2009).

Combined stress

Our results suggest the pathogen affects the stomatal control of Xynisteri, part of the strategies to tolerate drought. In plants with drought stress, the pathogen-inoculation at 1.5 dpi was associated with an additional rise in ABA. As expected, this resulted in a further decrease of the stomatal conductance in Chardonnay. In contrast, in drought-stressed Xynisteri, the pathogen was associated with slightly higher stomatal opening despite this pathogen-induced increase in ABA. In this cultivar, the combined stress also caused an additional rise in IAA, which has the ability to counteract ABA-induced closure (Pospíšilová 2003).

Moreover, the drought stress seemed to abolish the loss of chlorophyll by the pathogen stress. This indicates the chlorophyll loss only occurs as part of a successful infection by the pathogen and demonstrates the pathogen development at 1.5 dpi was already hindered compared to the fully irrigated plants. As would be expected when less chlorophyll is lost, the pathogen inoculation also decreased the loss of chlorophyll fluorescence due to prolonged drought stress in Chardonnay. In Xynisteri however, this loss was increased, despite the trends towards higher stomatal conductance and abolished chlorophyll loss. This is an indication that the response to the pathogen interferes with the adaptive strategies of Xynisteri to cope with drought stress, which are lacking in Chardonnay.

Oxidative stress parameters

Single drought stress

Plants generally respond to abiotic and biotic stresses with the production of ROS as signaling molecules. This is typically followed by the activation of the antioxidant system, to finely tune ROS-dependent signal transduction and prevent oxidative damage. During drought, the antioxidant system is activated sooner or stronger in a drought-tolerant than in a drought-sensitive cultivar (Laxa et al. 2019). ROS can severely damage many host cell components, by breaking DNA, destroying the function of proteins and causing lipid peroxidation (Harman et al. 1996). Lipid peroxidation is the most prominent symptom of oxidative stress in animals and plants (Yamamoto et al. 2001). It is highly correlated with the

concentration of MDA, one of its final products, which enhances cell membrane damage, leading to cell death, but also acts as a signaling molecule under stress conditions. Stress can disturb the well-maintained equilibrium between production and scavenging of ROS.

The drought-sensitive Chardonnay did not respond as fast to the drought stress as Xynisteri. In Chardonnay, the activity of the antioxidant enzymes only increased during prolonged drought stress. During the initial drought stress, the antioxidant enzymes in Chardonnay even showed lowered activity. Further illustrating the drought-sensitivity of Chardonnay, is the increasing loss of chlorophyll fluorescence, a measure for the maximum PSII quantum efficiency, and the gain in MDA as the drought stress prolonged. The indigenous cultivar Xynisteri on the other hand, is equipped with a basal tool set to cope with oxidative stress, including higher basal activity of the antioxidant enzymes and lower levels of H_2O_2. The drought-tolerant Xynisteri was able to handle the initial drought stress, by activating the antioxidant enzymes at an early stage, keeping H_2O_2 in balance. Xynisteri even demonstrated slightly higher chlorophyll content and chlorophyll fluorescence and reduced MDA levels compared to the fully irrigated control. However, this cultivar also suffered when the drought stress prolonged, with its oxidative responses becoming more similar to the responses of Chardonnay during initial drought stress.

Single pathogen stress

The single pathogen stress caused high lipid peroxidation, more than the drought stress, as indicated by the high correlation between MDA and pathogen-inoculated plants. Apart from ROS, increased lipoxygenase activity can also be the implicated in lipid peroxidation. Associated with JA biosynthesis, lipoxygenases are involved in the activation of defense signaling against *P. viticola* (Figueiredo et al. 2017). The course of oxidative stress can be observed particularly at 16 dot, when the H_2O_2 accumulation due to the pathogen infection at 1.5 dpi led to the highest accumulation of MDA. Interestingly, this was accompanied with strong decreases in antioxidative enzyme activity. The lipid peroxidation and weak oxidative burst during the first 24 hours of the compatible infection with *P. viticola* have been associated with slight increases of total antioxidant capacity (Figueiredo et al. 2017; Nascimento et al. 2019). The increased SA content might have inhibited the activities of the antioxidant enzymes, in order to enhance pathogenesis related (PR) gene expression (Klessig et al. 2000; Foyer and Noctor 2005). Inactivation of the antioxidant capacity to obtain stronger ROS production, could be key in boosting the plant defense and limiting the pathogen infection. Since sufficient oxidative burst can indeed restrain *P. viticola* (Figueiredo et al. 2017; Nascimento et al. 2019), the basally higher levels of H_2O_2 and the potentially SA-mediated, lower activity of antioxidant enzymes could be part of the more successful pathogen defense of Chardonnay.

However, despite the lowered activity of the antioxidant enzymes, ROS levels only increased slightly. Proline, which accumulated with MDA, could have been produced to quench and scavenge ROS, in order to stabilize proteins, DNA and membranes (Matysik et al. 2002; Hatmi et al. 2015). In the case of drought stress, proline rather than the antioxidant enzymes, has been associated with the detoxification of ROS in vines (Doupis et al. 2011). Previous studies have shown proline accumulated under stress by *P. viticola*

(Ali et al. 2012) and by drought (Doupis et al. 2011; Hatmi et al. 2015; Litskas et al. 2017; Tzortzakis et al. 2020). While the net impact of the host-pathogen interaction is clear, it is hard to make a distinction between host response, the pathogens modulation of this response and the pathogens biosynthesis. Brilli et al. (2018) report the *P. viticola* genome is holding the genes necessary for proline biosynthesis. It is possible that *P. viticola* might have impaired the oxidative burst by producing or triggering the production of proline, restricting ROS to small concentrations which are insufficient to restrain the pathogen.

Combined stress

The infection triggered similar losses of antioxidant enzyme activity in deficit as in fully irrigated plants. Both susceptible, fully irrigated and resistant, deficit-irrigated plants showed a dramatic proline accumulation. This indicates proline levels at 1.5 dpi can be a measure for the pathogen stress, whether infection was successful or not. The combined stress seemed to revert MDA and chlorophyll levels, although affected by both single stresses, to levels similar to non-stressed plants. This shows lipid peroxidation at 1.5 dpi mainly occurred during the successful infection by the pathogen and indicates the pathogen development was already hindered in the deficit-irrigated vines. The inoculation with the pathogen also seemed to mitigate the small changes in MDA and chlorophyll due to drought, an indication of the crosstalk between both responses.

The gap between in vitro **and** in planta **experiments**

Interestingly, depending on the inoculation occurring on leaf disks or intact plants, contradicting conclusions were reached about the impact of irrigation on the susceptibility to *P. viticola*. Because of the perennial nature and size of the grapevine plant, many studies investigating the impact of compounds, microorganisms, resistance genes or stress, are performed on detached leaves. Understandably, the cutting itself, but also the removal of the leaf from the elicitor of study and the plant system could trigger or inhibit responses in the leaf, resulting in responses different from those occurring *in planta*. *In vitro* studies of the plant response could oversimplify the system. This is especially the case when studying effects of abiotic stress, since placing the leaves in controlled conditions, partly relieves the excised leaf disks of the abiotic stresses the plants were experiencing. This study highlights the importance of being careful and critical in generalizing conclusions obtained through *in vitro* assays. While *in vitro* assays sometimes provide an excellent model, like the comparison of cultivar susceptibility under full irrigation, in other cases it proves impossible to extrapolate results of *in vitro* studies to the whole plant and field system.

Conclusions

Because of overlap and crosstalk between the responses to the individual stresses, the response to concurrent pathogen and drought challenge could not be interpolated from the independent stress response. Single drought stress triggered IAA and ABA, which antagonized JA and SA. Compared to the native cultivar Xynisteri, which boosted chlorophyll fluorescence and chlorophyll levels when initially faced with drought, the drought-sensitive Chardonnay activated the antioxidant system later and seemed

to suffer more as the drought stress prolonged. Chardonnay however, proved less susceptible to *P. viticola* than Xynisteri when irrigated. Under full irrigation, the successful infection by *P. viticola* at 1.5 dpi was associated with high IAA, SA and JA levels, strong decreases in antioxidant enzyme activity and parallel bursts in proline. When both stresses were combined, the response to the pathogen seemed to interfere slightly with the adaptive strategies of Xynisteri to cope with drought stress. Most interesting was the discovery that deficit irrigation induced resistance to this pathogen. Since drought-induced ABA overruled the SA and JA defense responses, generally implicated in the resistance against *P. viticola*, ABA is suggested to be involved in the resistance to *P. viticola*. Supporting this hypothesis is the additional rise in ABA observed in deficit-irrigated plants after inoculation with the pathogen, compared to non-inoculated plants. The nature of this ABA-mediated defense remains to be investigated, but is most likely post-invasive, since the changes in IAA, antioxidant enzyme activity and proline at 1.5 dpi occurred independent of the irrigation treatment. Other major findings in this study are the differences between concomitant and consecutive drought and pathogen stress, and as such between *in planta* and *in vitro* research. In sharp contrast to the *in planta* drought-induced resistance, leaves from drought-stressed plants became more susceptible to the pathogen when inoculated *in vitro*. This quick turn-over led us to conclude that high ABA concentrations are most important to the drought-induced resistance. It suggests that, once the ABA concentrations are lowered, the adverse effects of the drought on the pathogen response, such as the lowered JA, can increase susceptibility. The irrigation-dependent susceptibility highlights that the practices used to mitigate the effects of climate change, have a profound impact on plant pathogens. This study emphasizes that abiotic and biotic responses are closely intertwined and in the context of climate change, the impact on one should not be considered without the other. Since the timing of the exposure to the stresses can alter the outcome completely, it stands to question as to whether vines, under the studied field conditions, could become more vulnerable to *P. viticola* during a rain event following a drought period.

Declarations

Ethics Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Author's Contributions

This research was designed by MH, NT, AC and LH. NT and AC provided the infrastructure, plants and material for the execution of the experiment. The experiment was carried out by AC and LH, led by NT. AC determined the content of all oxidative parameters and chlorophyll. LH performed the infections and disease evaluations, with the support of MH, and the quantification of the phytohormones, with the expert guidance of KD. LH analyzed the data and wrote the original draft under supervision of MH. NT, AC and MH reviewed and edited the manuscript. All authors read and approved the published version of the manuscript.

Availability of Data and Materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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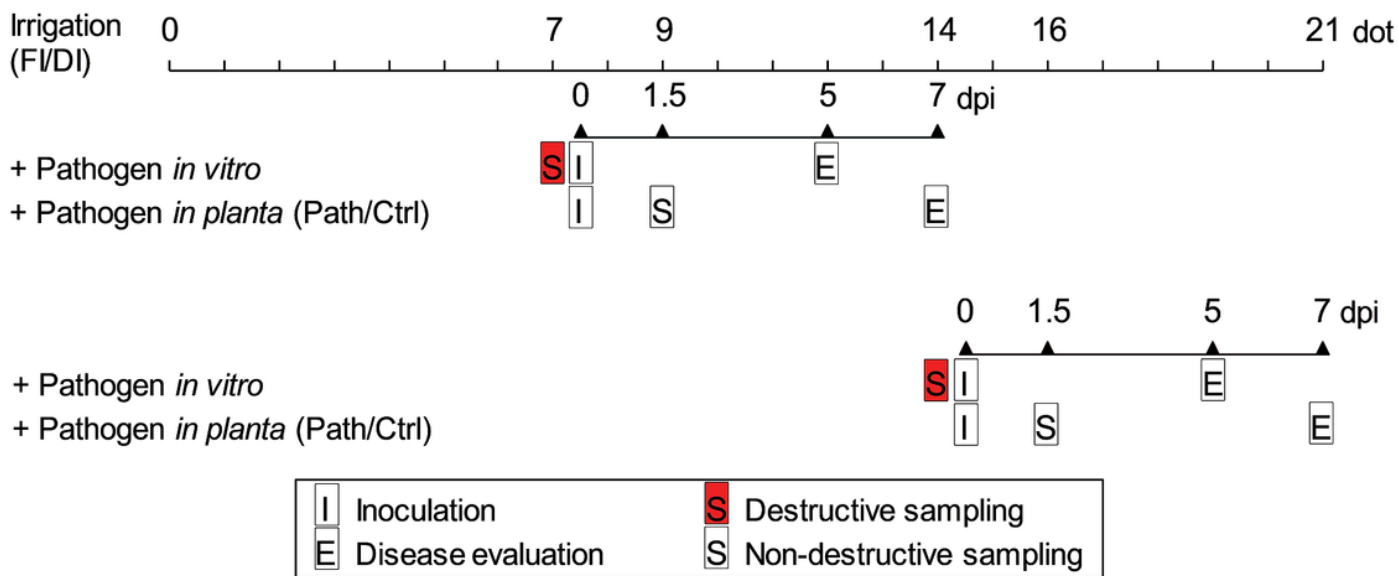
Figures

Figure 1

Scheme of the experimental setup. At 7 or 14 days of full (FI) or deficit (DI) irrigation, some plants were sampled destructively. Disks of these leaves were challenged with P. viticola in vitro. In the evening, the intact, remaining plants were inoculated with the pathogen (Path) or water (Ctrl). For these plants, the irrigation regime was maintained until disease evaluation. They were sampled at 1.5 dpi, corresponding with 9 or 16 dot. The disease evaluation of disks and plants was performed at 5 or 7 dpi, respectively. For each treatment, five replicates of Xynisteri and Chardonnay were used

Figure 2

Box plots representing basal hormone, chlorophyll and oxidative parameter levels in Xynisteri (XYN) and Chardonnay (CHAR). Leaves of five independent, fully irrigated plants, not inoculated with the pathogen, were analyzed. This was repeated at 7, 9, 14 and 16 dot, and the data were pooled. The levels of the phytohormones ABA, IAA, JA and SA are expressed in ng g-1 fresh weight (FW); chlorophyll a and b content in mg g-1 FW; H2O2 and proline content in µg g-1 FW; MDA content in nmol g-1 FW; activity of SOD, POD and CAT in units mg-1 protein. The parameters are considered significantly different for both cultivars if the p-value is below 0.05 (Mann-Whitney U-test). All observations are shown as dots. The line inside the box and the lower and upper boundary of the box represent the median, first and third quartile, respectively. The whiskers indicate the minimum and maximum, excluding outliers. All observations outside the 1.5 times interquartile range of the first or third quartile are considered outliers
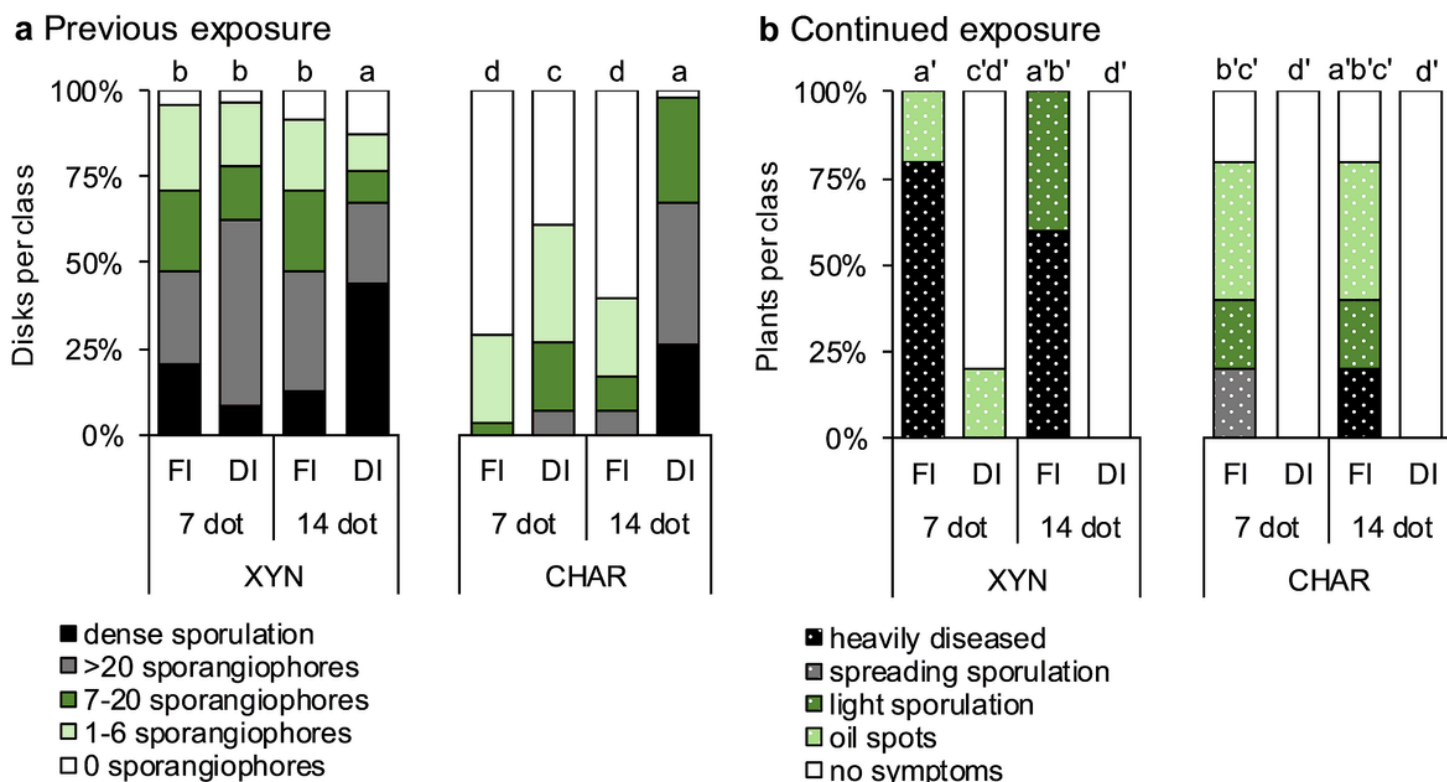
Figure 3

Influence of a previous and b continued exposure to drought stress on the susceptibility of Xynisteri (XYN) and Chardonnay (CHAR) to P. viticola. Control plants were fully irrigated (FI), while drought-stressed plants were exposed to deficit irrigation (DI). Different letters indicate significant differences (Mann-Whitney U-test; $p<0.05$). a Plants were exposed to 7 or 14 dot before detachment of the leaves. Leaf disks were inoculated with a P. viticola sporangia suspension and each disk was evaluated 5 dpi by counting the sporangiophores. Five plants of each cultivar were used per treatment, resulting in an average of 60 disks per treatment; b Plants were exposed to 7 or 14 dot and sprayed with a P. viticola sporangia suspension. The irrigation regime was maintained and disease severity was evaluated at 7 dpi. Five plants of each cultivar were used per treatment
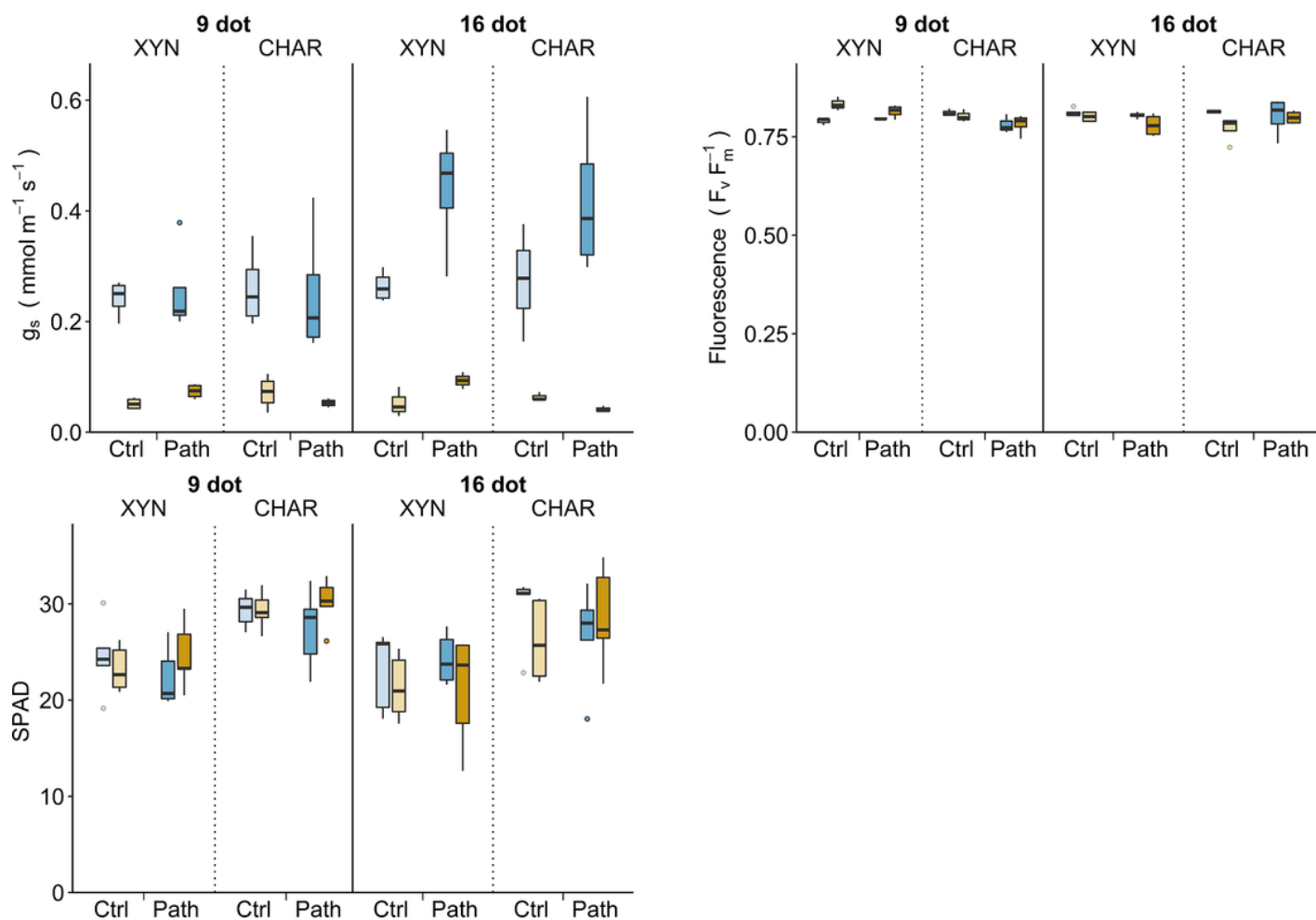
Figure 4

Box plots showing the impact of pathogen and drought stress on stomatal conductance, chlorophyll fluorescence and SPAD in Xynisteri (XYN) and Chardonnay (CHAR) at 9 and 16 dot. Plants had been subjected to 7 or 14 days of full (blue boxes; ⬜⬜) or deficit (yellow boxes; ⬜⬜) irrigation before inoculation with water (Ctrl) or P. viticola (Path). The line inside the box and the lower and upper boundary of the box represent the median, first and third quartile, respectively. The whiskers indicate the highest and lowest occurring value within the 1.5 times interquartile range. Outliers are marked as individual dots
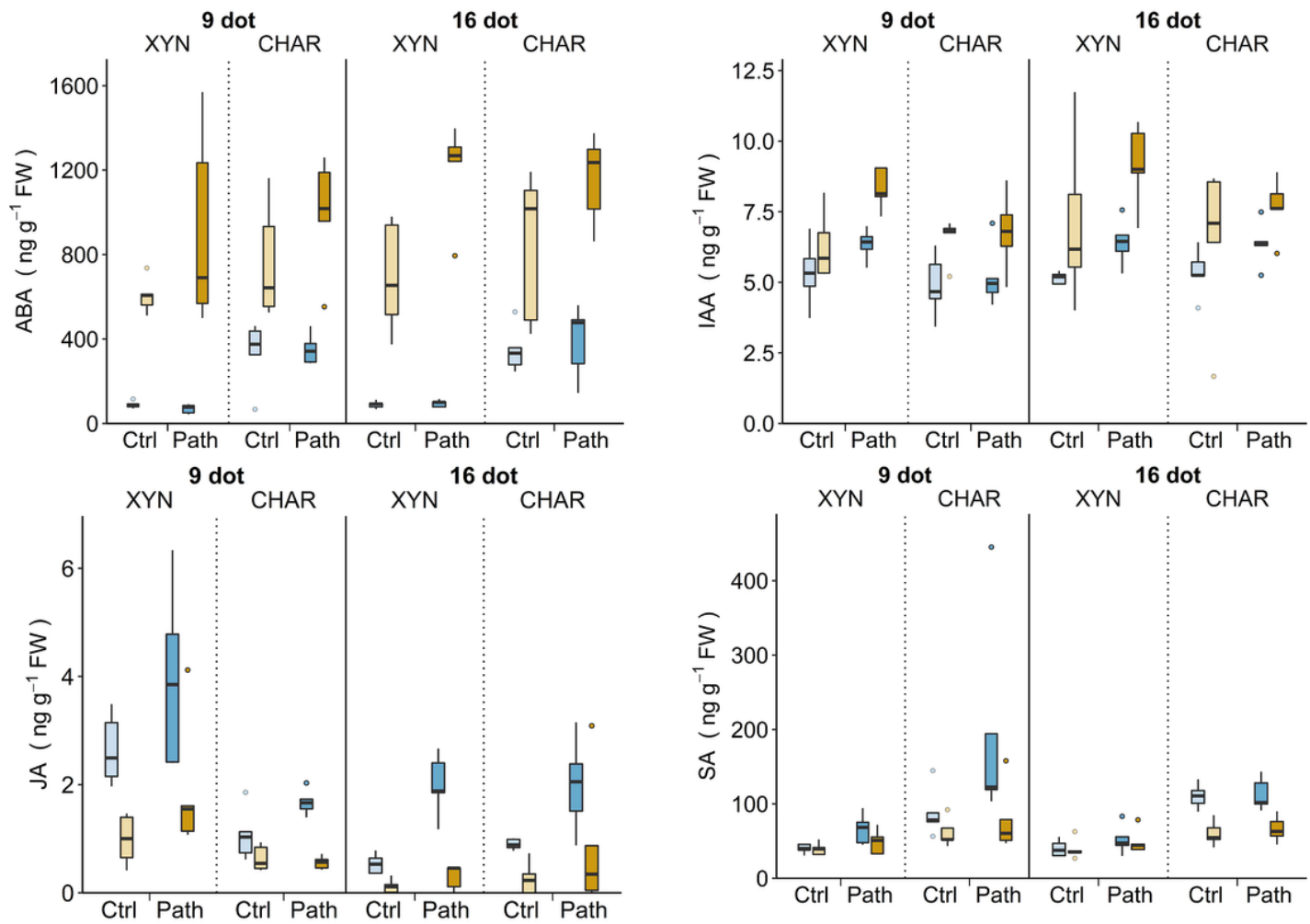
Figure 5

Box plots showing the impact of drought and pathogen stress on leaf phytohormone balance in the cultivars Xynisteri (XYN) and Chardonnay (CHAR). Plants had been subjected to 7 or 14 days of full (blue boxes; ▨▨) or deficit (yellow boxes; ▨▨) irrigation before inoculation with water (Ctrl) or P. viticola (Path). Samples for analysis were taken at 1.5 dpi, corresponding with 9 or 16 dot. Each treatment consisted of five repetitions. For the effect of drought stress, fully irrigated plants are to be compared with deficit-irrigated plants, for the effect of the pathogen stress water-inoculated (Ctrl) and pathogen-inoculated (Path) plants should be compared. The line inside the box and the lower and upper boundary of the box represent the median, first and third quartile, respectively. The whiskers indicate the highest and lowest occurring value within the 1.5 times interquartile range. Outliers are marked as individual dots
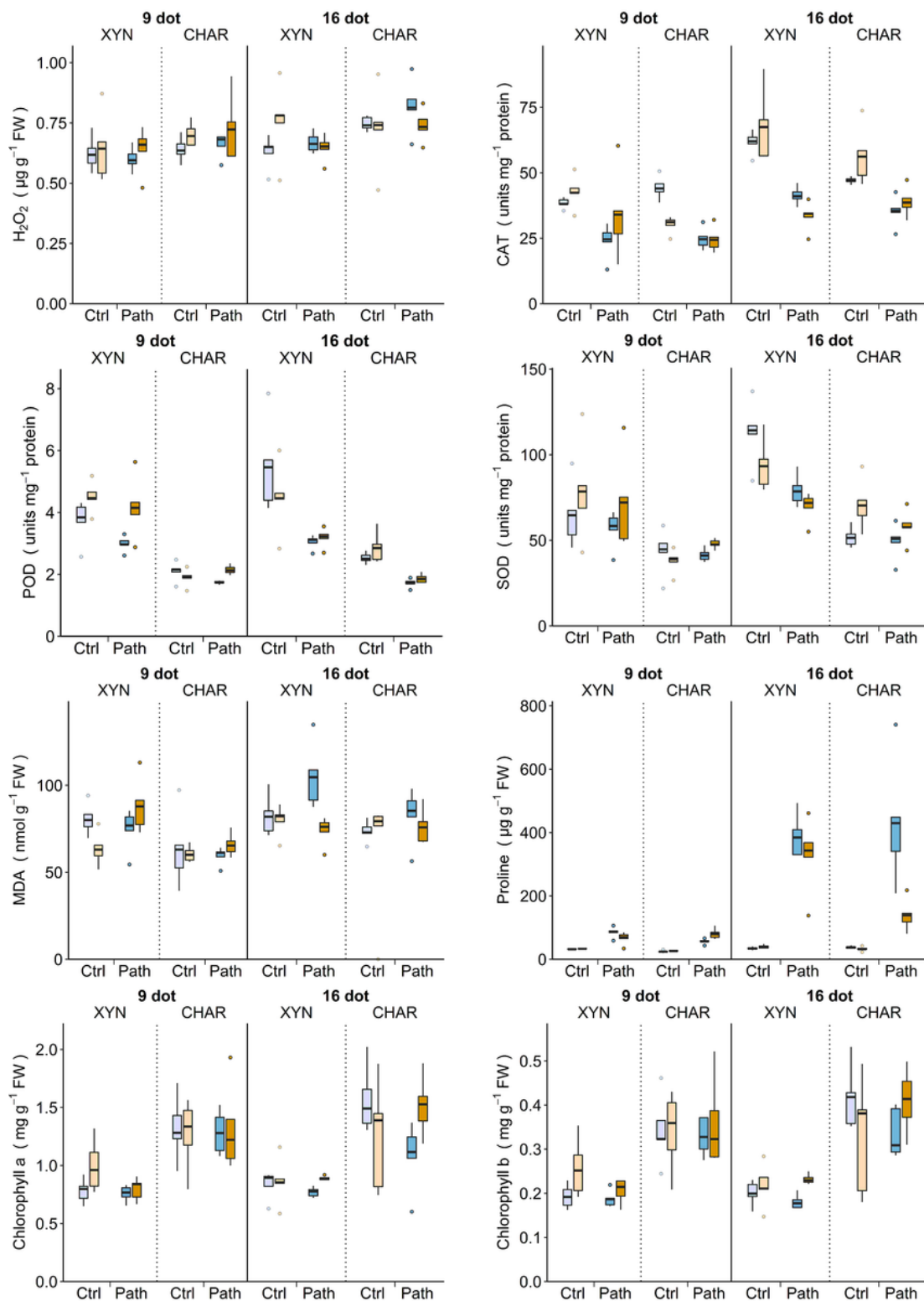
Figure 6

Box plots showing the impact of drought and pathogen on photosynthetic pigments and oxidative parameters in the cultivars Xynisteri (XYN) and Chardonnay (CHAR). Plants had been subjected to 7 or 14 days of full (blue boxes; 🟦) or deficit (yellow boxes; 🟨) irrigation before inoculation with water (Ctrl) or *P. viticola* (Path). Samples for analysis were taken at 1.5 dpi, corresponding with 9 or 16 dot. Each treatment consisted of five repetitions. To extract the effect of drought stress, full irrigation is to be

compared with deficit irrigation. The effect of the pathogen stress can be derived by comparing water-inoculated and pathogen-inoculated plants. The line inside the box and the lower and upper boundary of the box represent the median, first and third quartile, respectively. The whiskers indicate the highest and lowest occurring value within the 1.5 times interquartile range. Outliers are marked as individual dots
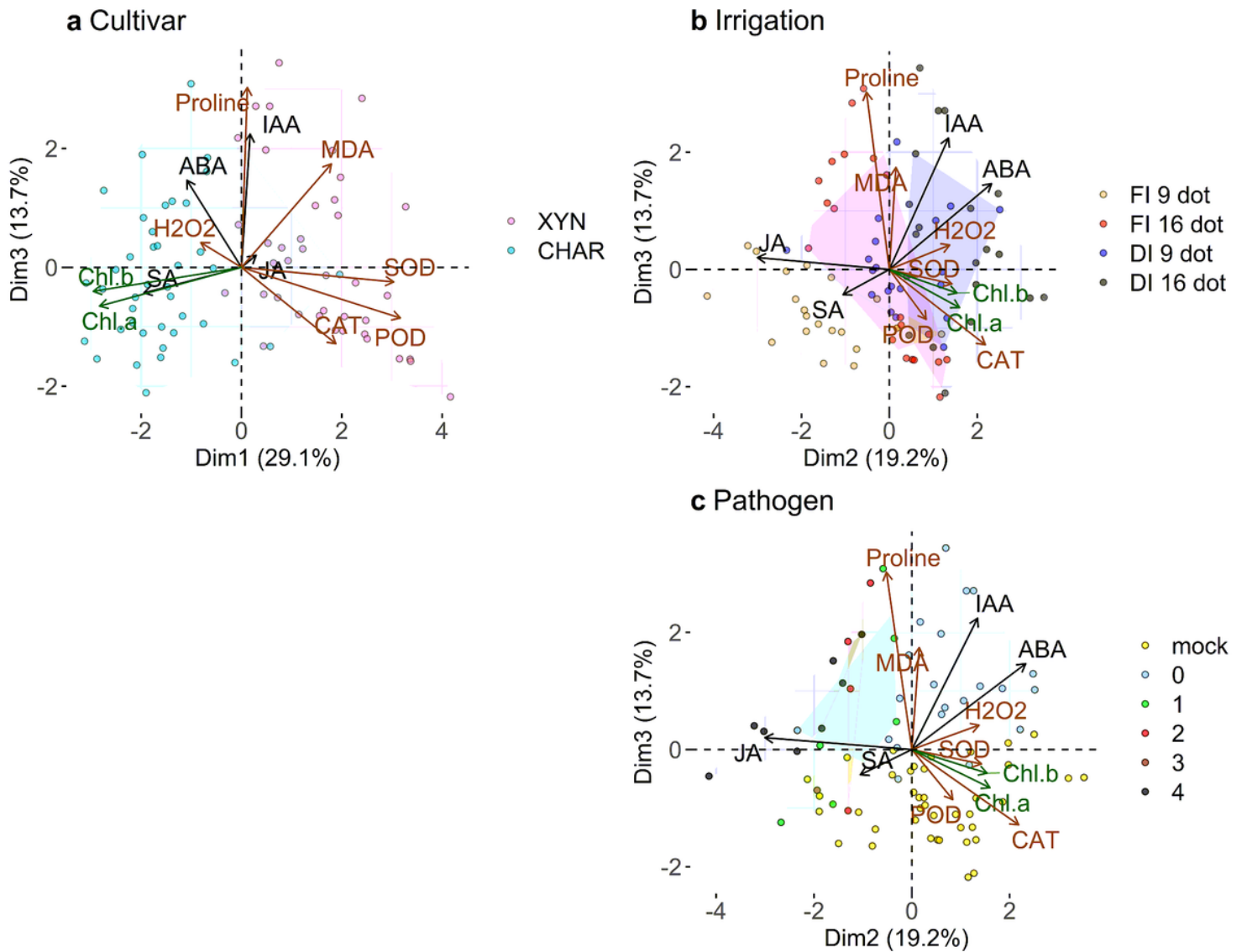

Figure 7

Principal component analysis visualizing the patterns in the phytohormone balance (black arrows), oxidative balance (brown arrows) and chlorophylls (green arrows). All plants are represented by dots. Grouping is performed according to a cultivar: drought-tolerant Xynisteri (XYN) or drought-sensitive Chardonnay (CHAR); b drought stress: full (FI) or deficit (DI) irrigation and duration (9 or 16 dot); c pathogen stress: water-inoculated (mock), no symptoms (0), oil spots (1), light sporulation (2), spreading sporulation (3), heavily diseased (4). The first, second and third principal component explain 29.1%, 19.2% and 13.7% of the variation respectively

Supplementary Files

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