

Responses to drought stress modulate the susceptibility to *Plasmopara viticola* in grapevine

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Abstract

Background

Climate change will increase the occurrence of plants being simultaneously subjected to drought and pathogen stress. Although it is well known that drought can alter the way in which plants respond to pathogens, knowledge about the effect of concurrent drought and biotic stress on 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 tolerances respond to challenge with *P. viticola*, drought stress or their combination and how one stress affects the other.

Results

Artificial inoculation was performed on two cultivars exposed to full or deficit irrigation in the Mediterranean climate of Cyprus. In parallel, leaf discs from these plants were inoculated under 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 cultivar Chardonnay. Successful infection by *P. viticola* at 1.5 days post inoculation was associated with high levels of indole-3-acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA), and proline and strong decreases in antioxidant enzyme activity. Drought, on the other hand, triggered the accumulation of IAA and abscisic acid (ABA), which antagonized JA and SA. Exposure to drought stress increased the susceptibility to *P. viticola* of the leaves inoculated under 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 of IAA, antioxidant enzyme activity, and proline still occurred in these drought-stressed plants. Surprisingly, ABA, rather than the generally implicated JA and SA, seemed to play a prominent role in this resistance.

Conclusions

Drought exposure increased the susceptibility of leaves inoculated *in vitro*. Conversely, deficit irrigation induced resistance to *P. viticola* in both Chardonnay and Xynisteri plants inoculated *in planta*. ABA, rather than JA and SA, was implicated in this resistance. The irrigation-dependent susceptibility indicates that the changing climate and the practices used to mitigate its effects may have a profound impact on plant pathogens.

Background

The global climate is changing. The rising temperatures, shifting precipitation patterns, and changing frequency of extreme weather events, such as droughts, are putting significant strain on many ecosystems [1]. Many viticultural areas are already suffering from seasonal drought, and water availability may become the bottleneck of wine production. The Mediterranean, a hotspot for wine

production, is one of the world's regions that is most vulnerable to the impact of climate change [2]. Global warming is very likely to aggravate the drying of soil in this region, increasing the intensity and frequency of drought episodes [2].

In the Eastern Mediterranean, the island of Cyprus is known for its hot, arid summers. Its mean annual precipitation is projected to decline by more than 20% by mid-century [3]. Viticulture has played an important role in Cyprus for over 5,500 years [4]. In 2017, vineyards covered 5% of the agricultural area [5]. With only 11% of the vine area under irrigation [5], most indigenous cultivars can be grown without irrigation. Drought tolerance has been an important selection criterion for these local grapevine cultivars. Since the end of the 20th century, however, the international market has 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 soils and the arid conditions in Cyprus [6].

The changing climate will also significantly affect the traits of plant pathogens, such as distribution, virulence, abundance, and host range [7, 8]. This will increase the likelihood of combined occurrence of drought and pathogen stress, which is already a very common event [9]. The complexity of the interactions between plants and pathogens gains another dimension with exposure to drought stress, resulting in a new state of stress [10]. Because of overlap and crosstalk between the responses to the individual stresses, this new stress will induce tailored responses customized to this specific stress combination [11]. Thus, the response to the concurrent challenge cannot simply be interpolated from the independent stress response [9, 11–15].

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 [16–19]. Concurrent drought stress most often aggravates disease [20, 21], but it can also trigger resistance [22, 23]. There has been much research on drought stress in grapevine, but less is known about pathogen stress, and there is almost no knowledge about the simultaneous occurrence of drought and pathogen stress [24]. A few studies provide information about consecutive, rather than simultaneous, drought and pathogen stress after inoculation of intact plants with *Plasmopara viticola* [25] or of detached leaves with *Botrytis cinerea* [26].

The current study aimed 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 [27, 28]. The interaction between both stresses is to be expected, since the stomata, the site of entry of *P. viticola*, are the plants' first line of defence against drought stress. *P. viticola* can manipulate stomatal behaviour [29], facilitating 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.

Results

Experimental set-up

The experimental setup is provided in Fig. 1. More details can be found in the Methods.

Basal differences between cultivars

Fig. 2 shows the basal levels of hormones, chlorophyll, and oxidative parameters in drought-tolerant Xynisteri and drought-sensitive Chardonnay plants without abiotic or biotic stress (control treatments for full irrigation; see Fig. 1). Notably, in Chardonnay, the abscisic acid (ABA) and salicylic acid (SA) levels were more than double, and the levels of chlorophyll a (Chl a) and chlorophyll b (Chl b) were almost double, the levels in Xynisteri. The H₂O₂ content tended to be slightly elevated in Chardonnay. In contrast, the peroxidase (POD) and superoxide dismutase (SOD) activities and the indole-3-acetic acid (IAA) and proline levels were significantly higher in Xynisteri than in Chardonnay. The malondialdehyde (MDA) content tended to be slightly higher in Xynisteri. For the catalase (CAT) activity and jasmonic acid (JA) content, no significant differences were observed between the cultivars.

Effect of drought stress on disease susceptibility

Previous exposure: in vitro inoculation

To assess the effect of previous exposure to drought stress on the susceptibility to *P. viticola*, Xynisteri and Chardonnay plants were first exposed to full or deficit irrigation, for either 7 or 14 days of irrigation treatment (dot), before *in vitro* inoculation of leaf discs with the pathogen (previous exposure to full/deficit irrigation; see Fig. 1). The disease evaluation results of the leaf discs are shown in Fig. 3a. When watered sufficiently (full irrigation - FI), Xynisteri showed significantly higher disease severity than Chardonnay. On discs from non-stressed Chardonnay plants, almost no sporulation was observed. However, Chardonnay leaf discs became more susceptible to *P. viticola* when exposed to 7 days of deficit irrigation (DI). On Xynisteri leaf discs, *P. viticola* was able to grow quickly, irrespective of exposure to drought stress. As the duration of previous drought exposure increased from 7 to 14 days, the disease severity increased in both cultivars. In particular, in Chardonnay, longer exposure to drought stress drastically enhanced susceptibility, reaching a level similar to that of Xynisteri.

Continued exposure: in planta inoculation

In parallel, the effect of continued exposure to drought stress on the susceptibility to *P. viticola* was examined. Chardonnay and Xynisteri plants were subjected either to 7 or 14 days of full or deficit irrigation before being sprayed with a *P. viticola* sporangial suspension or distilled water (continued

exposure to full/deficit irrigation; see Fig. 1). The disease evaluation results of the plants are shown in Fig. 3b. The irrigation regimen was maintained until the moment of disease evaluation, at 7 days post inoculation (dpi). Interestingly, under 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 plants challenged with a short drought stress period (7 days of deficit irrigation prior to inoculation) remained almost completely absent. Comparison of deficit-irrigated plants inoculated at 7 and 14 dpi showed that the resistance to *P. viticola* did not significantly change with prolonged exposure to deficit irrigation before inoculation.

Field measurements

Fig. 4 shows how drought and pathogen stress affect stomatal conductance and chlorophyll fluorescence. The statistical analyses of the single-stress treatments are indicated in Table 1. The physiological parameters were not significantly different between plants with and without pathogens for both irrigation regimens (statistics not shown).

Table 1 P-values for the single drought stress and single pathogen stress. Significant differences ($p \leq 0.05$) are highlighted in bold. This analysis was performed on subsets of the data to exclude interactions. For the effect of single drought stress, fully irrigated and deficit irrigated, water-inoculated plants were compared (control treatments of the continued exposure to full/deficit irrigation; see Fig. 1). For the effect of single pathogen stress, water-inoculated and pathogen-inoculated fully irrigated plants were compared (continued exposure to full irrigation; see Fig. 1).

	Effect of drought stress				Effect of pathogen stress			
	Xynisteri		Chardonnay		Xynisteri		Chardonnay	
Response variables	9 dot	16 dot	9 dot	16 dot	9 dot	16 dot	9 dot	16 dot
Stomatal conductance	0.029	0.057	0.029	0.050	0.886	0.057	0.686	0.200
Chlorophyll fluorescence	0.100	0.663	0.400	0.029	0.164	0.657	0.268	1.000
ABA	0.008	0.008	0.008	0.032	0.310	0.690	0.841	0.690
IAA	0.310	0.151	0.032	0.151	0.151	0.016	0.841	0.151
JA	0.008	0.008	0.095	0.012	0.222	0.008	0.095	0.056
SA	1.000	0.841	0.151	0.008	0.032	0.548	0.056	1.000
H ₂ O ₂	1.000	0.151	0.151	1.000	0.690	0.421	0.548	0.151
CAT	0.151	0.421	0.008	0.095	0.008	0.008	0.008	0.008
POD	0.056	0.690	0.310	0.310	0.151	0.008	0.151	0.008
SOD	0.421	0.222	0.310	0.032	0.548	0.016	0.310	1.000
MDA	0.032	1.000	1.000	0.310	0.690	0.032	0.690	0.151
Proline	0.310	0.151	0.222	0.151	0.008	0.008	0.008	0.008
Chl a	0.095	0.841	1.000	0.421	1.000	0.222	0.841	0.032
Chl b	0.151	0.548	1.000	0.421	0.841	0.310	1.000	0.095

Single drought stress

Drought stress had a profound effect on leaf stomatal conductance (Fig. 4, ç FI vs ç DI). After a short drought stress period (9 dot), the stomatal conductance had already plummeted in both cultivars. The chlorophyll fluorescence increased slightly in Xynisteri, but not in Chardonnay, after 9 days of deficit irrigation. Prolonged drought stress (16 dot) caused the chlorophyll fluorescence to decrease significantly in Chardonnay, but not in Xynisteri.

Single pathogen stress

Fig. 4 shows how the stomatal conductance and chlorophyll fluorescence measurements changed in response to *P. viticola* inoculation on fully irrigated plants (Fig. 4, ç FI vs ç FI + Path). No significant differences in the physiological parameters were observed between plants inoculated with water and plants inoculated with *P. viticola* for the same irrigation regimen (Table 1). However, an increasing trend in stomatal conductance due to pathogen infection was observed at 16 dot.

Combined stress

When both stresses were combined (Fig. 4, ζ DI + Path), the net effect on the physiological parameters was comparable to the strong effect of drought stress (Fig. 4, ζ DI). The physiological parameters were not significantly different between drought-stressed plants with and without the pathogen. Xynisteri exhibited a slightly higher stomatal conductance in plants under combined stress than in plants under single drought stress. Conversely, a slightly lower stomatal conductance was found in Chardonnay under combined drought stress.

Phytohormone balance

Fig. 5 shows how drought and pathogen stress affect the phytohormone content (continued exposure to full/deficit irrigation; see Fig. 1). The statistical analysis of the single-stress treatments is provided in Table 1. Interactions among the cultivar, pathogen stress, and type and duration of irrigation were analysed with a regression model (Additional file 1: Table S1, Additional file 2: Table S2).

Single drought stress

Short (9 dot) or prolonged (16 dot) deficit irrigation had a profound effect on the phytohormone balance (Fig. 5, ζ FI vs ζ DI). Drought stress caused significantly increased ABA and decreased JA levels in both cultivars, resulting in similar levels. Considering the large basal differences in ABA, Xynisteri produced much more ABA than Chardonnay in response to drought stress. Indeed, the ABA response to drought stress was likely cultivar dependent (Additional file 1: Table S1). Moreover, ABA levels seemed to increase when drought stress was prolonged. Drought stress was also associated with a slight increase in IAA in both cultivars. Finally, the SA response to drought stress was significantly dependent on the cultivar (Additional file 1: Table S1). Chardonnay responded to drought stress by decreasing its SA content, especially at 16 dot (Table 1), while still maintaining levels higher than those in 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. Hormone analysis was performed on samples taken at 1.5 dpi from fully irrigated plants (Fig. 5, ζ FI vs ζ FI + Path). Independent repetitions were conducted at 9 and 16 dot, with only a slight change in plant age. Although Xynisteri was more susceptible to *P. viticola* than Chardonnay (Fig. 3b), the hormonal responses of the cultivars to the pathogen were similar. Compared to water-sprayed plants, the pathogen-inoculated plants tended to accumulate more JA and IAA (Fig. 5). In the linear regression, there were indications that IAA was positively affected by the interaction with the pathogen, at least in Xynisteri (Additional file 2: Table S2). 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 regimen was maintained, and samples for analysis were taken at 1.5 dpi at 9 or 16 dot (Fig. 5, ç DI + Path). Interestingly, the ABA levels, which were already strongly increased by drought, increased even further 1.5 days after inoculation with the pathogen. The interaction between the pathogen and drought stress was highly significant for ABA (Additional file 1: Table S1). 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 in response to the pathogen was observed in drought-stressed plants, already demonstrating increased levels of IAA due to drought. A significant interaction between drought and pathogen stress was also observed for JA (Additional file 1: Table S1). The accumulation of JA in response to the pathogen, which occurred in fully irrigated plants, was not apparent when deficit irrigation was applied (Fig. 5).

Chlorophylls and oxidative balance

Fig. 6 demonstrates the impact of drought and pathogen stress on the chlorophyll content and oxidative parameters (continued exposure to full/deficit irrigation; see Fig. 1). The statistical analysis of the single-stress treatments is indicated in Table 1. The interactions among cultivar, pathogen stress, and irrigation type and duration were analysed with a regression model (Additional file 1: Table S1, Additional file 2: Table S2).

Single drought stress

To assess how short or prolonged drought stress affects 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 oxidative parameters (Fig. 6, ç FI vs ç DI). Even under drought stress, higher basal activities of SOD and POD in Xynisteri were maintained, just as the chlorophyll levels in Chardonnay remained significantly higher than those in Xynisteri.

Fig .6 shows differences in the responses of the cultivars to drought stress. In Xynisteri, the MDA content decreased significantly in response to a short drought stress (9 dot). Concurrently, the activities of the antioxidant enzymes (POD, SOD and CAT) and the chlorophyll content tended to be elevated. With prolonged drought stress (16 dot), the activities of the antioxidant enzymes POD and SOD seemed to diminish, and the H₂O₂ levels seemed to increase. Xynisteri's response no longer included changes in the MDA or chlorophyll content. In Chardonnay, on the other hand, there was no apparent influence of a short drought stress (9 dot) on the MDA or chlorophyll content. The activity of the antioxidant enzyme CAT dropped significantly (Table 1). Continued drought stress (16 dot) eventually caused an increase in the

activities of the antioxidant enzymes, significant for SOD (Table 1), while the chlorophyll content in Chardonnay seemed to decrease.

Single pathogen stress

To determine how the cultivars responded to *P. viticola*, the plants were sprayed with water or pathogen inoculum. The samples for analysis were taken from fully irrigated plants at 1.5 dpi (Fig. 6, ζ FI vs ζ FI + Path). In this case, both time points (9 or 16 dot) could be seen as repetitions, with only a small change in plant age. Despite the difference in disease severity between the cultivars (Fig. 3b), the responses of the cultivars to the pathogen were similar in terms of the parameters tested. What stood out most was the significant burst in proline associated with the pathogen-inoculated plants (Table 1). Additionally, the activities of the antioxidant enzymes (POD, SOD and CAT) were lowered in response to the pathogen stress. These responses were most pronounced at 16 dot, coinciding with a significant increase in the MDA content in Xynisteri and a significant decrease in the Chl a level in Chardonnay (Fig. 6).

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 regimen was maintained, and samples for analysis were taken at 1.5 dpi at 9 or 16 dot (Fig. 6, ζ DI + Path). Overall, the responses of deficit and fully irrigated plants to the pathogen were similar. The activities of the antioxidant enzymes CAT and POD were significantly reduced in pathogen-inoculated plants under full irrigation at 16 dot. Although no disease symptoms were seen on the drought-stressed plants (Fig. 3b), a reduction was also observed in plants subjected to drought and pathogen stress. Similarly, a clear increase in proline in pathogen-inoculated plants was observed under both irrigation regimens. This indicates that proline and the activities of the antioxidant enzymes were associated with inoculation rather than disease incidence.

The chlorophyll loss at 1.5 dpi in Chardonnay, on the other hand, might be associated with successful infection by *P. viticola*: in Chardonnay, the chlorophyll content seemed to increase with pathogen inoculation as drought was prolonged (16 dot), while it decreased under full irrigation (Fig. 6). Indeed, for Chl a, the interaction between abiotic stress, its duration, and biotic stress was significant (Additional file 1: Table S1). MDA also seemed to be strongly associated with successful infection: the MDA content increased in fully irrigated Xynisteri at 16 dot upon inoculation with the pathogen. Deficit-irrigated, inoculated plants at 16 dot exhibited levels of MDA similar to those in the noninoculated plants (Fig. 6). The MDA response to the pathogen depended primarily on the interaction with the cultivar, drought stress, and its duration (Additional file 1: Table S1).

Principal component analysis

Fig. 7 shows the principal component analysis (PCA) results for the continued exposure to full/deficit irrigation (Fig. 1). Although the cultivars and the irrigation and pathogen treatment were used as supplementary variables and did not participate in the construction of the dimensions, they allowed grouping of the data. A strong correlation was found between the first dimension (Dim1) and the cultivar (Table 3). Indeed, there was good separation between the two cultivars along the horizontal axis of the first dimension (Fig. 7a). The association with the first dimension indicates that the differences between the cultivars, both at the basal level 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 H₂O₂, while Xynisteri was associated with higher POD, SOD and CAT activities and MDA content (Table 3). The second dimension (Dim2) was mostly correlated with the irrigation regimen and, to a lesser extent, the duration of this treatment (Table 3). In the PCA, a shift is visible according to drought stress and its duration (Fig. 7b). Drought-stressed plants were mostly associated with ABA, IAA, and CAT activity but were also positively correlated with the chlorophyll and H₂O₂ levels and SOD and POD activities (Table 3). Fully irrigated plants were mainly correlated with JA and with SA to a lesser extent (Table 3). Finally, the third dimension was primarily correlated with pathogen stress (Table 3). In Fig. 7c, the water (Ctrl) and pathogen-inoculated plants showed clear separation along the vertical axis of the third dimension (Dim3), regardless of disease severity. Proline, IAA, and MDA seem to be strongly correlated and are associated with pathogen-inoculated plants.

Along the horizontal axis (Dim2) in Fig. 7c, the plants are separated according to disease severity, with symptomless plants on the right and the most diseased plants on the left. Upon clustering according to drought stress in the same dimensions (Fig. 7b), the group showing no symptoms overlapped with the drought-stressed group. Resistance was primarily observed in the 1st quadrant since a majority of the plants in the 3rd and 4th quadrants were not inoculated with the pathogen. The diseased plants were mainly clustered in the 2nd quadrant. The PCA suggests that JA is associated with infection under full irrigation. Interestingly, this PCA indicates that ABA and IAA play a role in drought-induced resistance, in which JA is no longer involved.

Table 2 Significant square correlation ratios (R^2) of the supplementary variables in the principal component analysis (PCA). R^2 indicates the strength of the correlation between the dimensions of the PCA and the variables cultivar (Chardonnay or Xynisteri), irrigation (full or deficit), duration (of the irrigation treatment; 9 or 16 dot), and pathogen (*P. viticola* or water inoculation). These supplementary variables were not involved in the construction of the dimensions. For each significant R^2 , the p-value is also shown.

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 3 Significant correlation coefficients of the active variables in the principal component analysis (PCA). The correlation coefficients describe the construction of the different dimensions of the PCA. For each significant coefficient, the p-value is also shown.

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 ₂ O 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	

Discussion

Phytohormones

Single drought stress

Single drought stress severely affected the phytohormone balance of the drought-tolerant cultivar Xynisteri and the drought-sensitive cultivar Chardonnay, which are native to and introduced into the investigated climate, respectively. Under these conditions, ABA, generally considered the key hormone underpinning mechanisms that regulate drought stress responses in plants, appeared to govern complex hormone crosstalk by antagonizing JA and SA. For both cultivars, drought stress increasingly triggered ABA as the duration increased but negatively impacted JA. The SA content was also lowered but primarily in the drought-sensitive cultivar Chardonnay. Although most studies showed that JA and SA are involved in drought stress responses in addition to ABA [30], the negative interaction of ABA with JA and SA has also been reported previously [31, 32]. Multiple nodes allow interference of ABA with the JA-ethylene pathway [33], but whether their interaction is antagonistic [33] or synergistic [34] strongly depends on the conditions. The suppressive effect of ABA on the SA signalling pathway [35–37] has been shown for grapevine, particularly by Wang et al. [38], who showed that elicitation with exogenous ABA led to a gradual reduction in SA.

Our data suggest that drought stress also caused the levels of IAA to increase in both cultivars. The higher basal IAA level 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 defence response against drought [39]. Through its crosstalk with reactive oxygen species (ROS), IAA can help plants adjust their growth to unfavourable conditions [40]. Previous studies have associated elevated auxin with the induction of abiotic stress-related genes, activation of the antioxidant response, and reduction in ROS accumulation [41–44].

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 that both high temperature [45, 46] and high light intensities [47] inhibit sporulation. Nights with minimum temperatures between 15 and 25°C and relative humidity (RH) 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 that 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 plant auxin signalling to suppress plant defences, while others can synthesize IAA themselves through various pathways to increase pathogenesis [48]. The IAA levels during *P. viticola* infection have not been studied previously, so the question of the origin and function of IAA accumulation remains. The accumulation of proline associated with elevated IAA has been observed in IAA-treated plants [49]. Similar to auxin, proline is also involved in numerous developmental processes [50], 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 an ROS

scavenger, proline might have been produced as part of the host defence 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 restricts pathogen development.

In contrast to abiotic stress, we found that pathogen stress acted positively on the JA and SA levels of both cultivars without an apparent effect on ABA levels. The increase in JA and SA at 1.5 dpi in infected, fully irrigated plants demonstrates that the plants were activating their defence mechanism. The basal level of SA was higher in Chardonnay and might be related to its more successful defence against *P. viticola*. The roles of JA and SA have been extensively studied in resistant cultivars, in which both phytohormones accumulate at high levels after infection with *P. viticola* [51]. SA- as well as JA-mediated defence responses are implicated in the resistance to *P. viticola* [52–56]. Moreover, exogenous JA has been shown to protect grapevine leaf discs against *P. viticola* through callose deposition [57]. The dynamics of endogenous phytohormones during compatible interactions with *P. viticola* have, however, not been explicitly investigated. Polesani et al. and Li et al. [52, 56] observed increases in JA, coupled with very strong increases in methyl jasmonate (MeJA), during successful infection. The endogenous levels of both hormones increased from 12 to 48 h post inoculation but were eliminated once the tissue was completely invaded [52], indicating the involvement of JA and MeJA in defence in the early developmental stages of the pathogen in compatible interactions.

Combined stress

The effect under combined abiotic and biotic stress was completely different. When 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. Interestingly, the JA and SA levels were low and no longer substantially contributed to the pathogen defence response. Our results reveal that under concurrent stress, ABA dominated the responses to pathogen stress occurring under full irrigation, antagonizing JA and SA. Furthermore, a significant additional increase in ABA was observed in the inoculated compared to the noninoculated 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*. However, how might ABA contribute to the inhibition of infection by *P. viticola*?

Considered a global regulator of plant stress responses, ABA is crucial in the response of plants to multiple stresses [11]. Its role in pathogen defence is poorly understood. Whether ABA acts as a positive or negative regulator of disease resistance is dependent on the stage of infection and the pathosystem but seems to be unrelated to the pathogen lifestyle or mode of attack [16]. Although most studies have established an antagonistic relationship between ABA and disease resistance [33, 35, 58–60], treatment of detached grapevine leaves with exogenous ABA has been shown to result in a reduction in *P. viticola* infection, albeit only at high concentrations [57, 61].

ABA can be involved in preinvasive defence, preventing pathogen penetration by controlling rapid stomatal movement [62]. Our data suggest, however, that the pathogen was not blocked completely during preinvasive defence. Despite their differences in disease susceptibility, both fully and deficit-irrigated pathogen-inoculated plants showed major changes in IAA and proline levels and CAT, POD, and, to a lesser extent, SOD activities. Their independence from the irrigation treatment at this infection stage (1.5 dpi) indicates that the infection in the deficit-irrigated plants ceased post penetration. This result indicates that *P. viticola* was able to penetrate the substomatal cavities, even though the stomatal conductance was markedly reduced in response to deficit irrigation. Notably, an additional increase in ABA was observed in deficit-irrigated plants after inoculation with the pathogen. This additional increase in ABA could be key to postinvasive resistance to this pathogen. During postinvasion defence, ABA is involved in callose [34, 63] and stilbene [38] accumulation, thus limiting pathogen spread. ABA has also been found to accumulate strongly in some genetically resistant *Vitis* species after *P. viticola* inoculation [38, 64]. In many resistant *Vitis* species, most infections never advance beyond the assessed developmental stage (24-48 hpi) [52, 65].

However, while continued exposure to drought induced resistance, we discovered that leaves detached from drought-stressed plants became more susceptible to this pathogen when inoculated in humid, temperate conditions. This indicates that drought-induced resistance depends on a rapid defence response, which can be reversed in a very short time. The fast turnover of drought-induced resistance could explain why Roatti et al. [25] 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 that a physical barrier would be the source of ABA-mediated, postinvasive resistance. Regardless, the response seems to be strongly dependent on the ABA concentration, which is determined by ABA production, transport, and catabolism. The rate at which ABA is catabolized might be proportional to the amount of ABA accumulated [66]. Hence, once drought stress is lifted and ABA is no longer synthesized, high levels of ABA cannot be sustained. We hypothesize that the recovered disease susceptibility in the detached leaves of drought-stressed plants is linked to their inability to maintain sufficiently high ABA levels and to restore the adverse effects of drought on pathogen defence in a timely manner. After all, drought severely interfered with the pathogen response, including inducing IAA and antioxidant enzyme activity and antagonizing JA and SA levels. From this point of view, it is not surprising that post drought, Chardonnay partially lost its high tolerance to the pathogen. The *in vitro* susceptibility of this cultivar even increased with the duration of the previous drought stress. In addition to the increased adverse effects, additional ABA accumulation occurred when deficit irrigation was prolonged. This potentially caused lower ABA levels post drought as a result of the increased ABA catabolism. Previous exposure to deficit irrigation also deteriorated Xynisteri's pathogen defence, 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 a sudden threat to 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, thereby inducing

resistance, but for the introduced cultivar, irrigation is of greater importance. The increasing carbon footprint, coupled with the additional irrigation and disease control measures, underlines the growing importance of the “right plant, right place” concept. 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 adaptations to quickly cope with water stress and might have less sensitive stomatal control than Xynisteri [67, 68]. The higher basal concentrations of ABA in the leaves of Chardonnay compared to those of Xynisteri might be related to anisohydric behaviour [69]. As a native cultivar in Cyprus, Xynisteri has likely developed rapid mechanisms to avoid drought stress. At 9 days of deficit irrigation, a slightly higher chlorophyll fluorescence (a measure of the maximum photosystem II quantum efficiency) and chlorophyll content were observed in Xynisteri, compared to full irrigation. Eventually, at 16 dot, the losses in chlorophyll fluorescence and chlorophyll content seemed higher in Chardonnay. This might be an indication that drought was a greater burden on Chardonnay than on Xynisteri.

Single pathogen stress

In plants without drought stress, *P. viticola* was able to infect its hosts proficiently by manipulating them during infection. Particularly at 16 dot, the pathogen seemed to increase stomatal conductance, potentially as a result of the accumulation of IAA after infection [70], since the ABA levels were not substantially lowered. It is well known that *P. viticola* is able to manipulate stomatal movements. Stoll et al. [71] reported that stomatal conductance in irrigated plants decreased under infection with *P. viticola*, while other studies observed that the pathogen kept the stomata open by suppressing ABA production [72], by degrading ABA, or by blocking ABA transport [29]. The infection also slightly decreased the chlorophyll content, although the chlorophyll fluorescence did not appear to be affected. This biotroph has been shown to lower the photosynthetic rate [73, 74] through the loss of chlorophyll; downregulation of the chlorophyll a/b binding protein, chlorophyll synthase, and Rubisco; and upregulation of chlorophyllase [75]. Only 2 days after inoculation, the chlorophyll losses recorded were still small, likely because the chlorophyll content decreased only within the infected lesion [75, 76] and might still have been insufficient to affect chlorophyll fluorescence [77].

Combined stress

Our results suggest that the pathogen affects the stomatal control of Xynisteri, part of the strategy to tolerate drought. In plants under drought stress, pathogen inoculation at 1.5 dpi was associated with an additional increase in ABA. As expected, this resulted in a further decrease in stomatal conductance in Chardonnay. In contrast, in drought-stressed Xynisteri, the pathogen was associated with a slightly higher stomatal opening despite this pathogen-induced increase in ABA. In this cultivar, the combined stress also caused an additional increase in IAA, which has the ability to counteract ABA-induced closure [70]. Moreover, drought stress seemed to abolish the loss of chlorophyll caused by pathogen stress in Chardonnay. This indicates that chlorophyll loss only occurred as a part of successful infection by the pathogen and demonstrates that pathogen development at 1.5 dpi was already hindered compared to that of the fully irrigated plants. Conversely, pathogen inoculation also decreased the loss of chlorophyll content and chlorophyll fluorescence due to prolonged drought stress, in Chardonnay. This might be an indication of the crosstalk between the responses to pathogen and drought stress.

Oxidative stress parameters

Single drought stress

Plants generally respond to abiotic and biotic stresses with the production of ROS as signalling molecules. This is typically followed by 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 cultivar than in a drought-sensitive cultivar [78]. ROS can severely damage many host cell components by damaging DNA, destroying the functions of proteins, and causing lipid peroxidation [79]. Lipid peroxidation is the most prominent symptom of oxidative stress in animals and plants [80]. It is highly correlated with the concentration of MDA, one of its final products, which enhances cell membrane damage, leading to cell death. MDA also acts as a signalling molecule under stress conditions. Stress can disturb the well-maintained equilibrium between the production and scavenging of ROS.

The drought-sensitive Chardonnay did not respond as fast to 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. Chardonnay exhibited an increasing loss of chlorophyll fluorescence as drought stress was prolonged. The indigenous cultivar Xynisteri, on the other hand, is equipped with a basal toolset to cope with oxidative stress, including higher basal activity of antioxidant enzymes and lower levels of H_2O_2 . The drought-tolerant cultivar Xynisteri was able to tolerate the initial drought stress by activating antioxidant enzymes at an early stage and maintaining the H_2O_2 balance. Xynisteri even demonstrated slightly higher chlorophyll content and chlorophyll fluorescence and significantly reduced MDA levels compared to the fully irrigated control. However, this cultivar was also affected when drought stress was prolonged, with its oxidative responses becoming more similar to the responses of Chardonnay during initial drought stress.

Single pathogen stress

Single pathogen stress caused high lipid peroxidation, more than drought stress, as indicated by the high correlation between MDA and pathogen-inoculated plants. In addition to ROS, increased lipoxygenase activity is also involved in lipid peroxidation. Associated with JA biosynthesis, lipoxygenases are involved in the activation of defence signalling against *P. viticola* [74]. The course of oxidative stress could be observed, particularly at 16 dpi, when H₂O₂ accumulation due to pathogen infection at 1.5 dpi led to the highest accumulation of MDA. Interestingly, this was accompanied by strong decreases in antioxidative enzyme activity. Lipid peroxidation and weak oxidative burst during the first 24 hours of compatible infection with *P. viticola* have been associated with slight increases in total antioxidant capacity [74, 81]. The increased SA content might have inhibited the activities of the antioxidant enzymes to enhance pathogenesis-related (PR) gene expression [82, 83]. Inactivation of the antioxidant capacity to obtain stronger ROS production could be key in boosting plant defence and limiting pathogen infection. Since a sufficient oxidative burst can indeed restrain *P. viticola* [74, 81], the higher basal levels of H₂O₂ and the potentially SA-mediated, lower activity of antioxidant enzymes could be a part of the more successful pathogen defence strategy of Chardonnay.

However, despite the lowered activity of the antioxidant enzymes, H₂O₂ levels increased only slightly. Proline could have been produced to quench and scavenge ROS to stabilize proteins, DNA, and membranes [26, 84]. In the case of drought stress, proline, rather than antioxidant enzymes, has been associated with the detoxification of ROS in vines [85]. Previous studies have shown that proline accumulates under stress caused by *P. viticola* [86] and by drought [6, 26, 85, 87]. While the net impact of the host-pathogen interaction is clear, it is difficult to distinguish among host response, pathogen modulation of this response, and pathogen biosynthesis. Brill et al. [88] reported that the *P. viticola* genome contains the genes necessary for proline biosynthesis. Therefore, *P. viticola* might have impaired the oxidative burst by producing or triggering the production of proline, restricting ROS to small concentrations that are insufficient to restrain the pathogen.

Combined stress

The infection triggered similar losses of antioxidant enzyme activity in the deficit-irrigated and fully irrigated plants. Both susceptible, fully irrigated and resistant deficit-irrigated plants showed strong proline accumulation. This indicates that proline levels at 1.5 dpi can be a measure of pathogen stress, whether the infection is successful or not. The combined stress seemed to revert the MDA levels and chlorophyll levels, although affected by the single pathogen stress, to levels similar to those in nonstressed plants. This shows that lipid peroxidation at 1.5 dpi mainly occurred during successful infection by the pathogen and indicates that pathogen development was already hindered in the deficit-irrigated vines. Finally, a short drought led to slightly higher chlorophyll fluorescence and chlorophyll levels and significantly lower MDA levels in Xynisteri, compared to the fully irrigated control. Concurrent pathogen inoculation reduced these initial responses of Xynisteri to drought. This might be 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.

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

Interestingly, depending on the inoculation occurring on leaf discs or intact plants, contradictory conclusions were reached regarding 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, as well as the removal of the leaf from the elicitor of interest 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 the effects of abiotic stress, since placing the leaves in controlled conditions partly relieves the excised leaf discs of the abiotic stresses that the plants were experiencing. This study highlights the importance of being careful and critical in generalizing conclusions obtained through *in vitro* assays. Sometimes, *in vitro* assays provide an excellent model, such as for the comparison of cultivar susceptibility under full irrigation. In other cases, extrapolation of the results of *in vitro* studies to the whole plant and field system proves impossible.

Conclusions

Because of overlap and crosstalk between the responses to the individual stresses, the response to the 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 be more strongly affected with the prolongation of drought stress. Chardonnay, however, was less susceptible to *P. viticola* than Xynisteri when irrigated. Under full irrigation, 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 interestingly, deficit irrigation induced resistance to this pathogen in both Chardonnay and Xynisteri. Since drought-induced ABA overruled the SA and JA defence responses, generally implicated in resistance against *P. viticola*, ABA is suggested to be involved in this resistance to *P. viticola*. Consistent with this hypothesis was the additional increase in ABA observed in deficit-irrigated plants after inoculation with the pathogen compared to noninoculated plants. The nature of this ABA-mediated defence remains to be investigated but is most likely postinvasive, since the changes in IAA, antioxidant enzyme activity, and proline at 1.5 dpi occurred independently 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 turnover led us to conclude that high ABA concentrations may be most important to drought-induced resistance. This suggests that once ABA concentrations are lowered, the adverse effects of drought on the pathogen response, such as lowered JA, can increase susceptibility to *P. viticola*. It still stands to question whether vines, under the studied field conditions, could become more vulnerable to *P. viticola* during a rain event following a drought period.

The irrigation-dependent susceptibility highlights that the practices used to mitigate the effects of climate change may have a profound impact on plant pathogens. For sustainable vineyard management, the effect of deficit and full irrigation on crops and prevailing plant pathogens should be evaluated. To avoid downy mildew epidemics, the application of deficit rather than full irrigation may be advisable when drought becomes intolerable. In arid conditions, however, deficit irrigation might not be sufficient for introduced cultivars. The increasing carbon footprint associated with the additional irrigation and disease control measures of the introduced cultivar underlines the growing importance of the “right plant, right place” concept. In the context of climate change, the impact on one stress should not be considered without the other. Breeding programmes, breeding for the future, should pay special attention to the combination of biotic and abiotic stresses. The expected increase in abiotic stress might also be of importance in selecting resistance-inducing beneficial microorganisms or elicitors since abiotic stress might interfere with the pathways needed to trigger resistance. All of these studies must, however, take into consideration that a simplified model, such as the leaf disc model, cannot be used without prior comparison with the whole-plant model.

Methods

Site description and plant material

This research was conducted in 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. Additional file 3: 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 to 24% RH. On an average night, the minimum temperature dropped to 19.5°C, and the RH reached 70%.

This study included two cultivars of *Vitis vinifera*, namely, Xynisteri and Chardonnay. Xynisteri is the main white grape cultivar grown in Cyprus, while Chardonnay, one of the most planted white grape cultivars internationally, was introduced in Cyprus. In 2014, Xynisteri and Chardonnay covered 30.2% and 1.6% of the ca. 6,142 ha viticultural area of Cyprus, respectively [5]. Sixty self-rooted cuttings of each cultivar were planted in 5-litre polyethylene pots containing soil originating from the traditional vineyard area in Limassol. The soil properties were previously described by Tzortzakis et al. [87]. Briefly, the soil had a clay-loam texture, an organic matter content of 2.19%, a total CaCO₃ content of 66.9%, a pH of 7.42, and an electrical conductivity (EC) of 0.28 of mS cm⁻¹. The plants were grown in field conditions and

automatically irrigated at field capacity using a drip irrigation system. Three months after planting, the plants were uniformly distributed over 12 treatment groups. The experimental setup is shown in Fig. 1. For each treatment, five replicates were used per cultivar. 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 dpi. *In vitro* inoculations were performed on discs of these leaves. In the evening, the remaining intact plants were inoculated with either pathogen or water. For these plants, the irrigation regimen was maintained until disease evaluation 7 days later. Some leaves were sampled at 9 and 16 dpi to establish the effect of pathogen attack at 1.5 dpi.

Abiotic stress

Plants were either well-watered, in the full irrigation treatment, or exposed to drought stress by deficit irrigation. The 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 (Additional file 4: Fig. S2).

Biotic stress

To examine the combined effect of abiotic and biotic stress on the vine, pathogen stress was imposed on the intact plants after 7 or 14 days of drought stress (continued exposure to full irrigation; see Fig. 1). *P. viticola* isolate FCHPV1, 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. Artificial inoculation was performed in the evening. The abaxial sides of all leaves were sprayed until run-off with a 3 mL sporangial suspension of *P. viticola*. The control plants were sprayed with distilled water. The irrigation regimens were maintained until disease evaluation. Since *P. viticola* needs 95-100% RH during the night for optimal infection and sporulation, each plant was equipped with a container of water and a moist 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 were conducted at 1.5 and 7 dpi, respectively. Each plant was evaluated according to the following classification: 0, no symptoms; 1, few oil spots with little to no sporulation; 2, moderate symptoms and nonspreading sporulation; 3, clearly diseased with spreading sporulation; and 4, severe symptoms with dense sporangiophore carpets.

Field measurements

At 9 and 16 dot, the stomatal conductance and chlorophyll fluorescence 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 vapour was measured on three to five plants using a transient state diffusion porometer (AP4; Delta-T Devices). The chlorophyll fluorescence ($F_v F_m^{-1}$), 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).

In vitro assessment of disease susceptibility

The 3rd and 4th leaves, counted from the apex, sampled at 7 and 14 dot (previous exposure to full irrigation; see Fig. 1), were used to investigate the effect of the previous exposure to drought stress on the susceptibility to *P. viticola*. Leaf discs (11 mm diameter) were treated with 20 μ L of distilled water or 20 μ L of *P. viticola* sporangial 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, more than 20 sporangiophores; and 4, numerous sporangiophores. An average of 60 discs was evaluated per treatment.

Quantification of phytohormones

In leaves sampled at 7, 9, 14, and 16 dot, the levels of ABA, IAA, JA, and SA were determined, in ng per g fresh weight (FW). 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 the quantification of phytohormones is described in detail by Haeck et al. [89]. The ground tissue (100 mg) was incubated with 5 mL of 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 of methanol/water/formic acid (20:80:0.1, v/v/v). Chromatographic separation was performed on an ultra-high performance liquid chromatography system (UHPLC, Thermo Fisher Scientific) equipped with a Nucleodur C18 column (50 \times 2 mm; 1.8 μ m 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 μ L 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 the 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%; and 10-14 min at 20%. External and deuterated internal (d_4 -SA at $200 \mu\text{g L}^{-1}$, d_6 -ABA and d_5 -IAA at $1 \mu\text{g L}^{-1}$) 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. The leaf tissue (100 mg) was incubated in a heat bath at 65°C for 30 min with 10 mL of 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 the Chl a and Chl b concentrations were calculated as described by Richardson et al. [90].

Quantification of the hydrogen peroxide content, lipid peroxidation, and proline content

For quantification of the hydrogen peroxide (H_2O_2) content, lipid peroxidation (in terms of the MDA content), and the proline content, 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, the 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 [91]. For 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 1 M potassium iodide (KI). The H_2O_2 content was calculated using standards of 5 to 500 μM 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} .

The proline content was also determined using the frozen ground tissue. The leaf tissue (200 mg) was homogenized in 2 mL of 3% aqueous sulfosalicylic acid (SSA). The 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 a blank. The proline concentration was determined using serial dilutions (0-100 $\mu\text{g mL}^{-1}$) of D-proline [92].

Quantification of antioxidant enzymes

The ground leaf samples were also used for determination of the activity of the antioxidant enzymes. The tissue (200 mg) was homogenized with 3 mL of ice-cold 50 mM PPB (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 [93], with bovine serum albumin (BSA) as the protein standard.

The CAT (EC 1.11.1.6) activity was determined by following the consumption of H₂O₂ (extinction coefficient 39.4 mM cm⁻¹) at 240 nm for 3 min, as assayed by Jiang and Zhang [94]. The reaction mixture contained 100 mM PPB (pH 7.0), plant extract, and 200 µL of 75 mM H₂O₂. The results are expressed as CAT units per milligram of protein. One unit of enzyme decomposed 1 µmol of H₂O₂ per min.

The SOD (EC 1.15.1.1) activity was assayed using a 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. The 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. The reaction without the extract developed maximal colour (control), and a nonirradiated 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 for 50% inhibition of the NBT photoreduction rate [95].

The POD (EC 1.11.1.7) activity was determined according to the method used by Tarchoune et al. [96]. The POD activity was assayed using pyrogallol, following the increase in absorbance at 430 nm, after oxidation to purpurgallin. The 2-mL reaction mixture 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 H₂O₂. The increase in absorbance at 430 nm was measured on a kinetic cycle for 3 min. Calculations were performed using 2.47 mM cm⁻¹ as the coefficient of extinction. One POD unit was defined as the amount of enzyme needed to decompose 1 µmol of H₂O₂ per min.

Statistical analysis

All statistical analyses were conducted using R, version 3.6.1 [97]. The disease evaluation results were analysed using the Kruskal-Wallis test, followed by the Mann-Whitney *U*-test (*p*=0.05). For comparison of the other parameters between treatment groups, normality and homoscedasticity were first checked with Shapiro-Wilk's and Levene's tests (*p*=0.05). Since the data did not meet the conditions of normality and homogeneity of variances, the results were analysed using the Kruskal-Wallis test, followed by the Mann-Whitney *U*-test (*p*=0.05). For analysis of the interactions among the cultivar, drought, and pathogen stress,

a 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.

Abbreviations

ABA: abscisic acid; AIC: Akaike information criterion; ANOVA: one-way analysis of variance; BSA: bovine serum albumin; CAT: catalase; Chl a: chlorophyll a; Chl b: chlorophyll b; DMSO: dimethyl sulfoxide; dot: days of irrigation treatment; dpi: days post inoculation; EC: electrical conductivity; EDTA: ethylenediaminetetraacetic acid; ET: ethylene; FW: fresh weight; HSD: honestly significant difference; IAA: indole-3-acetic acid; JA: jasmonic acid; MDA: malondialdehyde; NBT: nitro blue tetrazolium; PCA: principal component analysis; PMSF: phenylmethylsulfonyl fluoride; POD: peroxidase; PPB: potassium phosphate buffer; PR: pathogenesis related; PVPP: polyvinylpolypyrrolidone; RH: relative humidity; ROS: reactive oxygen species; SA: salicylic acid; SOD: superoxide dismutase; SSA: sulfosalicylic acid; TBA: thiobarbituric acid; TCA: trichloroacetic acid; TDR: time-domain reflectometer; UHPLC: ultra-high performance liquid chromatography; VWC: volumetric water content

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in Additional file 5: Table S3.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

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

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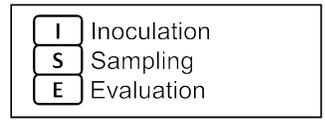
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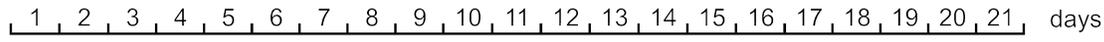
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Figures



FULL IRRIGATION



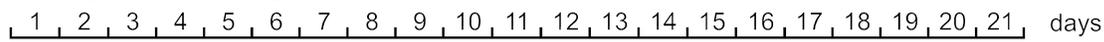
Short full irrigation



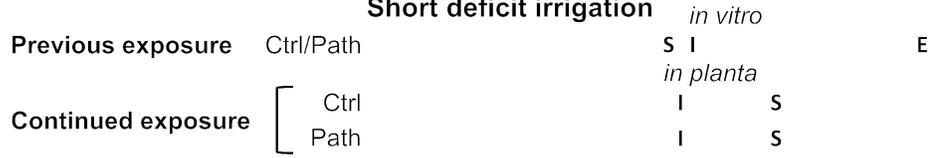
Prolonged full irrigation



DEFICIT IRRIGATION



Short deficit irrigation



Prolonged deficit irrigation



Figure 1

The twelve treatments used in this experimental setup. Each treatment group, represented by one timeline, consisted of five plants of both cultivars: drought-resistant Xynisteri and drought-sensitive Chardonnay. The plants were first exposed to 7 days of full/deficit irrigation (short full irrigation/short deficit irrigation) to establish the effect of short-term drought stress or to 14 days of full/deficit irrigation (prolonged full irrigation/prolonged deficit irrigation) to examine the effect of prolonged drought stress. At day 7 or 14, the plants were either sampled destructively or were maintained under the current irrigation regimen. Some of the sampled leaves of the first group were used for in vitro inoculation with water (Ctrl) or P. viticola (Path) to assess the effect of previous exposure to drought stress on disease development. The other groups were inoculated in planta, either with water (Ctrl) or with P. viticola (Path), to determine the effect of continued exposure to drought stress on disease development. These plants were sampled at 9 or 16 days of irrigation treatment (dot), corresponding to 1.5 days post inoculation (dpi), and were maintained under full/deficit irrigation until disease evaluation at 7 dpi.

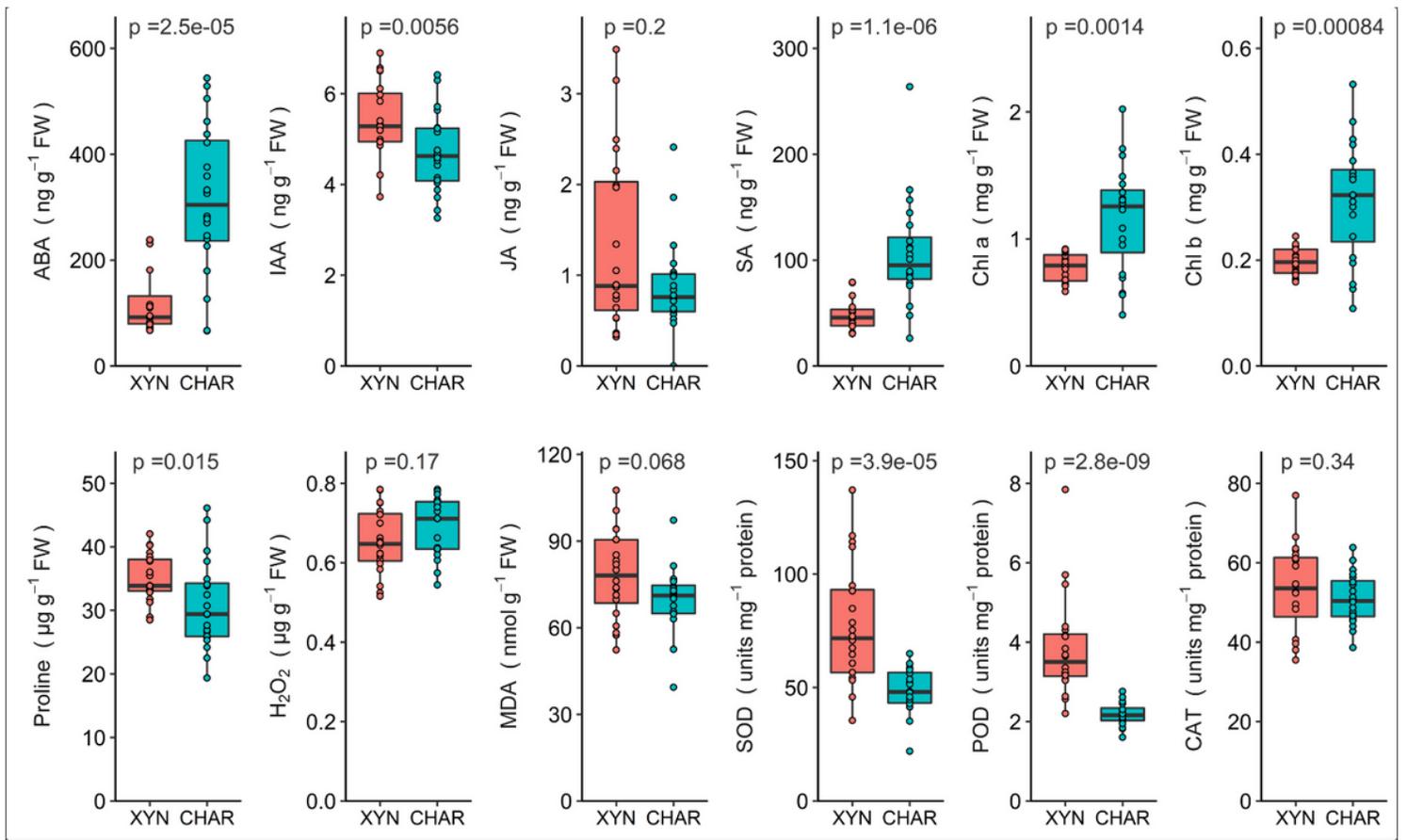


Figure 2

Basal levels of hormones, chlorophyll, and oxidative parameters in Xynisteri (XYN) and Chardonnay (CHAR). Samples from fully irrigated plants that were not inoculated with the pathogen were analysed and pooled. The samples were collected from five plants of each cultivar at 7 and 14 days of full irrigation (previous exposure to full irrigation, before inoculation; see Fig. 1) and at 9 and 16 days of full irrigation (control treatments of continued exposure to full irrigation; see Fig. 1). The parameters are considered to be significantly different for both cultivars if the p-value is below 0.05 (Mann-Whitney U-test). All observations are shown as dots. The lines inside the box and the lower and upper boundaries of the box represent the median and first and third quartiles, respectively. The whiskers indicate the minimum and maximum values, excluding outliers. All observations outside the 1.5-fold interquartile range of the first or third quartile are considered outliers.

a Previous exposure: *in vitro* inoculation

b Continued exposure: *in planta* inoculation

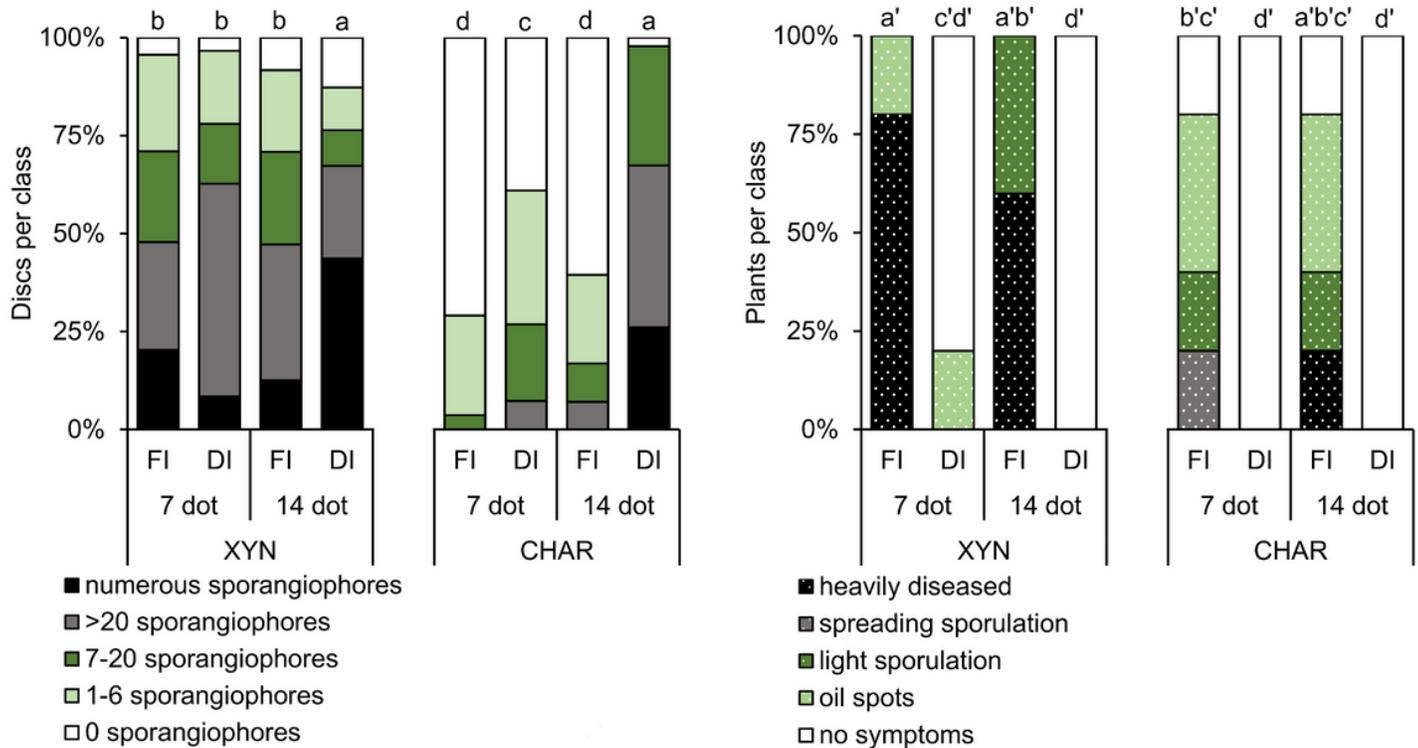


Figure 3

Influence of drought stress on the susceptibility of Xynisteri (XYN) and Chardonnay (CHAR) to *P. viticola*. The control plants were fully irrigated (FI), while the drought-stressed plants were exposed to deficit irrigation (DI). Different letters indicate significant differences (Mann-Whitney U-test; $p=0.05$). a Previous exposure to drought stress: Plants were exposed to 7 or 14 dot before the leaves were detached (previous exposure to full/deficit irrigation; see Fig. 1). Leaf discs were inoculated with a *P. viticola* sporangial suspension, and each disc was evaluated 5 days later by counting the sporangiophores. Five plants of each cultivar were used per treatment, resulting in an average of 60 discs per treatment. b Continued exposure to drought stress: Plants were exposed to 7 or 14 dot prior to being sprayed with a *P. viticola* sporangial suspension (continued exposure to full/deficit irrigation; see Fig. 1). The irrigation regimen was maintained until the disease severity was evaluated 7 days later. Five plants of each cultivar were used per treatment.

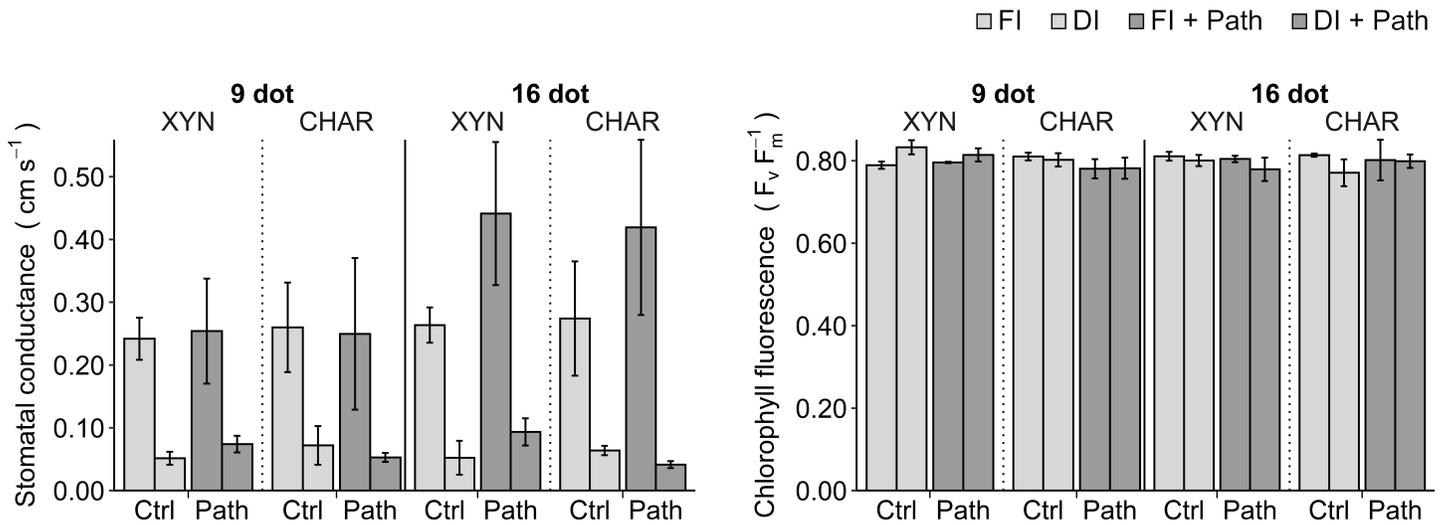


Figure 4

The impact of drought and pathogen stress on plant physiological parameters. Xynisteri (XYN) and Chardonnay (CHAR) plants were subjected to 7 or 14 days of full (FI) or deficit (DI) irrigation before in planta inoculation with water (Ctrl) or *P. viticola* (Path) (continued exposure to full/deficit irrigation; see Fig. 1). The stomatal conductance and chlorophyll fluorescence were measured at 1.5 days post inoculation (dpi), corresponding to 9 or 16 days of irrigation treatment (dot). For the effect of drought stress, the fully irrigated and deficit-irrigated control plants should be compared. For the effect of pathogen stress, water-inoculated and pathogen-inoculated fully irrigated plants should be compared. The bars and error bars show the mean and the standard deviation, respectively.

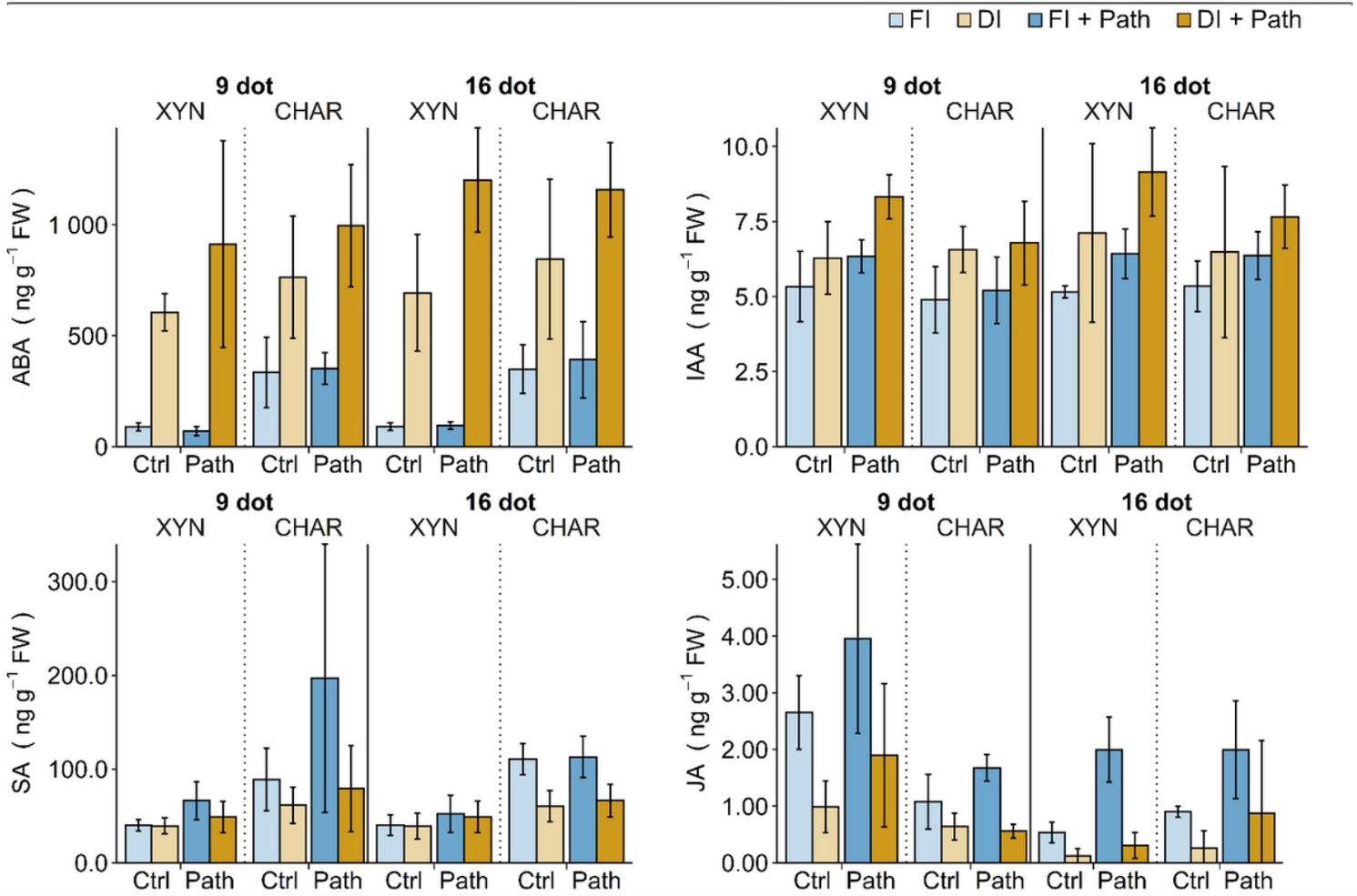


Figure 5

The impact of drought and pathogen stress on the leaf phytohormone balance. Xynisteri (XYN) and Chardonnay (CHAR) plants were subjected to 7 or 14 days of full (FI) or deficit (DI) irrigation before in planta inoculation with water (Ctrl) or *P. viticola* (Path) (continued exposure to full/deficit irrigation; see Fig. 1). Samples for analysis were taken at 1.5 days post inoculation (dpi), corresponding to 9 or 16 days of irrigation treatment (dot). Each treatment consisted of five repetitions. For the effect of drought stress, the fully irrigated and deficit-irrigated control plants should be compared. For the effect of pathogen stress, water-inoculated and pathogen-inoculated fully irrigated plants should be compared. The bars and error bars show the mean and the standard deviation, respectively.

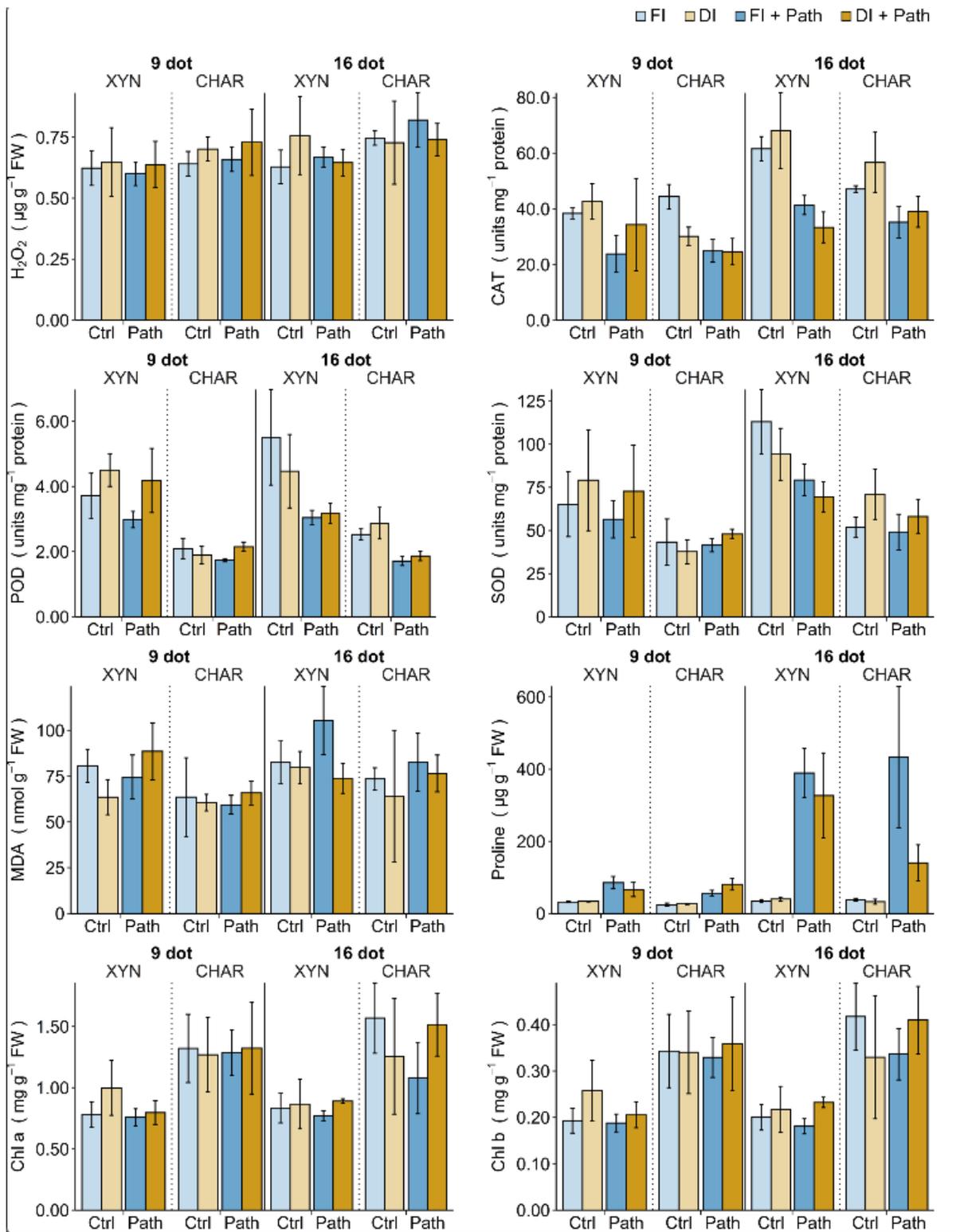


Figure 6

The impact of drought and pathogen stress on photosynthetic pigments and oxidative parameters. Xynisteri (XYN) and Chardonnay (CHAR) plants were subjected to 7 or 14 days of full (FI) or deficit (DI) irrigation before in planta inoculation with water (Ctrl) or *P. viticola* (Path) (continued exposure to full/deficit irrigation; see Fig. 1). Samples for analysis were taken at 1.5 days post inoculation (dpi), corresponding to 9 or 16 days of irrigation treatment (dot). Each treatment consisted of five repetitions.

For the effect of drought stress, the fully irrigated and deficit-irrigated control plants should be compared. For the effect of pathogen stress, water-inoculated and pathogen-inoculated fully irrigated plants should be compared. The bars and error bars show the mean and the standard deviation, respectively.

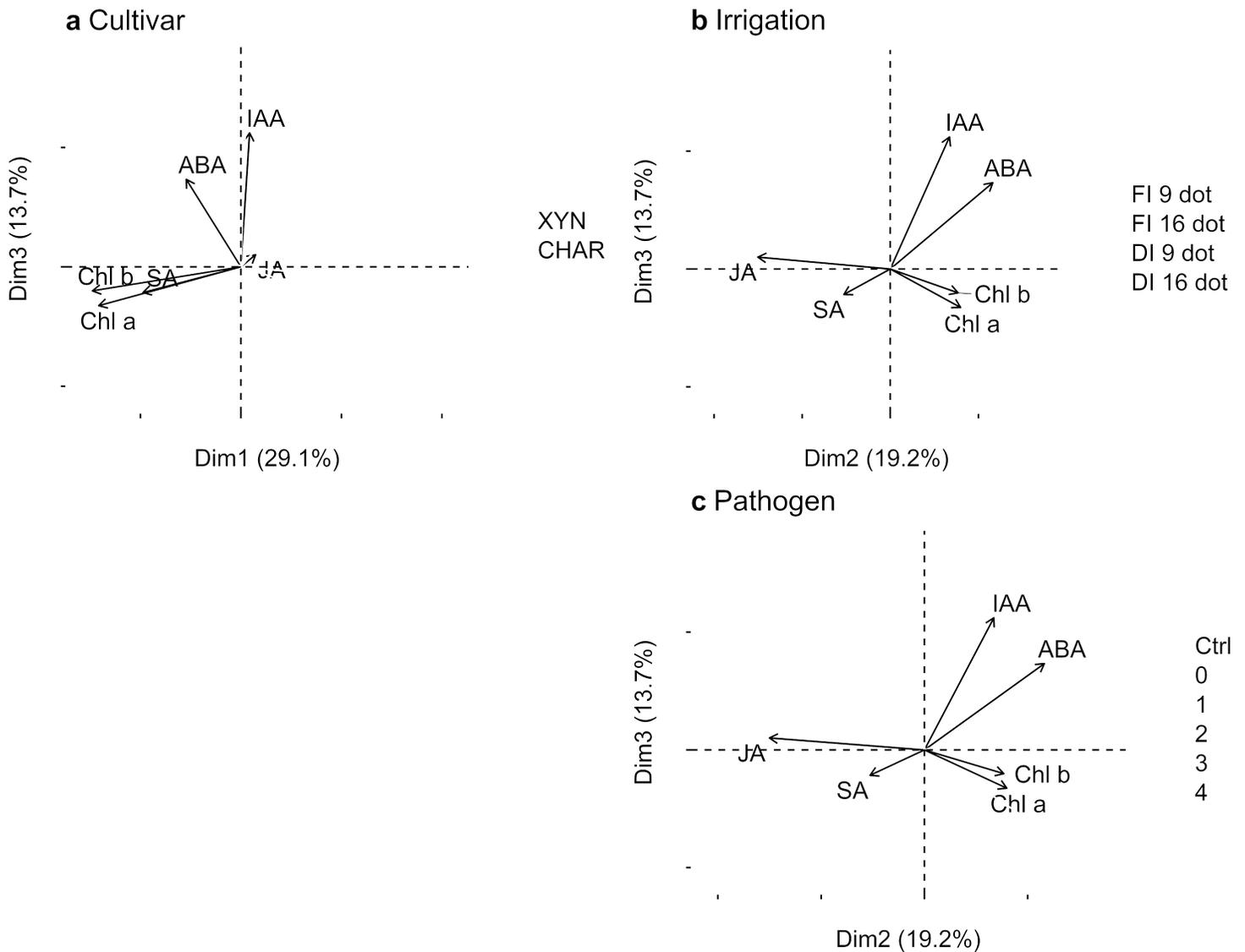


Figure 7

Principal component analysis visualizing the patterns in the phytohormone balance, oxidative balance and chlorophyll content. Phytohormones are indicated with black arrows, oxidative parameters with brown arrows, and chlorophyll with green arrows. All plants (treated with continued exposure to full/deficit irrigation; see Fig. 1) 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, at 9 or 16 days of irrigation treatment (dot); c pathogen stress: water-inoculated (Ctrl), no symptoms (0), oil spots (1), light sporulation (2), spreading sporulation (3), heavily diseased (4). The first, second, and third principal components explained 29.1%, 19.2%, and 13.7% of the variation, respectively.

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