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Research paper

Mechanisms of induced susceptibility to *Diplodia* tip blight in drought-stressed Austrian pine

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Plants experiencing drought stress are frequently more susceptible to pathogens, likely via alterations in physiology that create favorable conditions for pathogens. Common plant responses to drought include the production of reactive oxygen species (ROS) and the accumulation of free amino acids (AAs), particularly proline. These same phenomena also frequently occur during pathogenic attack. Therefore, drought-induced perturbations in AA and ROS metabolism could potentially contribute to the observed enhanced susceptibility. Furthermore, nitrogen (N) availability can influence AA accumulation and affect plant resistance, but its contributions to drought-induced susceptibility are largely unexplored. Here we show that drought induces accumulation of hydrogen peroxide (H_2O_2) in Austrian pine (*Pinus nigra* Arnold) shoots, but that shoot infection by the blight and canker pathogen *Diplodia sapinea* (Fr.) Fuckel leads to large reductions in H_2O_2 levels in droughted plants. In in vitro assays, H_2O_2 was toxic to *D. sapinea*, and the fungus responded to this oxidative stress by increasing catalase and peroxidase activities, resulting in substantial H_2O_2 degradation. Proline increased in response to drought and infection when examined independently, but unlike all other AAs, proline further increased in infected shoots of droughted trees. In the same tissues, the proline precursor, glutamate, decreased significantly. Proline was found to protect *D. sapinea* from H_2O_2 damage, while also serving as a preferred N source in vitro. Fertilization increased constitutive and drought-induced levels of some AAs, but did not affect plant resistance. A new model integrating interactions of proline and H_2O_2 metabolism with drought and fungal infection of plants is proposed.

Keywords: fungal pathogen, *Pinus nigra*, proline, reactive oxygen species, water stress.

Introduction

Water deprivation is perhaps the most important source of stress for trees. Drought conditions affect trees both directly, by inducing impairment of water relations, and indirectly, by making trees more susceptible to biotic attacks, e.g., from pathogens (Desprez-Loustau et al. 2006, Sturrock et al. 2011). Although drought-induced tree susceptibility to pathogens is a well-known phenomenon, the underlying molecular and physiological mechanisms remain poorly understood.

Plants have evolved a variety of shared adaptations to cope with drought, perhaps explaining why drought stress is such a common predisposing factor for disease (Schoeneweiss 1975, Chaves et al. 2003, Bhargava and Sawant 2013). For instance, droughted plants typically experience greater oxidative stress (Cruz de Carvalho 2008), which can lead to the production of free radicals and reactive oxygen species (ROS) (Apel and Hirt 2004, Pospíšil 2009). Free radicals and ROS are extremely reactive and can be especially damaging to critical cellular components, including DNA, lipids, membranes, proteins and other

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cellular machinery (Mittler 2002). Thus, their generation and regulation must be tightly controlled.

Solute accumulation is another common drought-related response in many plant species (Hare et al. 1998). In particular, studies have shown that the accumulation of free proline is one of the most conserved processes and that this amino acid (AA) functions as an osmoregulator and compatible solute (Delauney and Verma 1993, Hare and Cress 1997, Szabados and Savouré 2010). Proline can also act as a substitute electron acceptor, making it capable of scavenging free radicals, which can stabilize subcellular structures and protect enzymes (Schobert and Tschesche 1978, Hare and Cress 1997), a feature not found in all stress-induced solutes (Smirnoff and Cumbes 1989). Proline may also remove metal ions capable of redox activity, which helps prevent hydroxyl radical formation (Miller et al. 2010). Through its controlled synthesis and catabolism, proline can also regulate cellular redox homeostasis, which is perturbed during stress, by serving as an electron acceptor and regenerating cofactors (Kishor et al. 2005). Furthermore, once a stress event is over, proline can serve as a carbon and nitrogen (N) source to fuel recovery (Kishor et al. 2005, Szabados and Savouré 2010). These advantageous functions are likely the reason why proline accumulation is also observed in response to other abiotic stressors, including those that would seem to lack a clear osmotic component, such as exposure to heavy metals (Alia and Saradhi 1991), low and high temperatures (Chu et al. 1978), anaerobic conditions (Chen 1980) and air pollution (Anbazhagan et al. 1988). In addition to proline, glutamate plays a pivotal role in plant responses to abiotic stress, because it serves as the major precursor to proline and other important amine metabolites [e.g., γ-aminobutyric acid (GABA)] (Rhodes et al. 1986, 1999, Kinnersley and Turano 2000), and is a central hub for N metabolism, assimilation and transport (Lam et al. 1996, Kim et al. 1999, Seifi et al. 2013*b*).

Several plant responses to abiotic stress are shared with biotic stress (Cruz de Carvalho 2008, Verslues and Sharma 2010), and can conceivably influence the outcome of plant–pathogen interactions. For instance, ROS are often produced during infection and can be directly toxic to invading organisms, but are also involved in defensive signaling pathways (Wojtaszek 1997, Bolwell and Daudi 2009, Torres 2010). Proline frequently accumulates during biotic stress as well (Hare and Cress 1997, Verbruggen and Hermans 2008, Rojas et al. 2014), though its precise role in defense is not clear in most systems. However, in *Arabidopsis* and tobacco, proline metabolism is thought to play an important role in innate immunity against non-host pathogens, by triggering the hypersensitive response [HR, a type of programed cell death (PCD)] via the pyrroline-5-carboxylate (P5C)-proline cycle (Fabro et al. 2004, Cecchini et al. 2011, Senthil-Kumar and Mysore 2012, Monteoliva et al. 2014). Like proline, glutamate and its metabolites are purported to be critical elements of plant defense strategies (Seifi et al. 2013*a*, 2013*b*). Thus, it is clear that AA and ROS metabolism are prominent in adaptation to both abiotic and biotic stress (Wojtaszek 1997, Cheeseman 2007, Slesak et al. 2007, Bolton 2009), and may be an important link between drought stress and predisposition to pathogens.

The AA content of plants is affected by N availability (Sauberlich et al. 1953, Kim et al. 1987, Näsholm and Ericsson 1990, Näsholm and McDonald 1990). Therefore, N availability may influence the outcome of plant–pathogen interactions through changes in AA composition. The interactive effects of drought and N availability have not been extensively studied; however, N availability can affect the drought-induced accumulation of proline (Díaz et al. 2005, Wang et al. 2011). While it is clear that drought and N availability alter AA metabolism, the molecular aspects of the relationship with plant defense have not been extensively investigated in trees, and thus it is not known whether drought and N supply interactively affect plant resistance or how they alter the plant's molecular profile.

We tackled the question of how drought stress predisposes trees to fungal pathogens by focusing on interactions between Austrian pine (*Pinus nigra* Arnold) and *Diplodia sapinea* (Fr.) Fuckel (Phillips et al. 2013). This pathogen is widespread and results in tip blight and canker diseases of two-needled pines. These diseases are greatly exacerbated by drought, in Austrian as well as other pines (Blodgett et al. 1997*b*, Stanosz et al. 2001, Desprez-Loustau et al. 2006). We hypothesized that (i) drought induces proline and glutamate accumulation, providing *D. sapinea* with an important source of nutrients and protection from ROS and (ii) N fertilization amplifies these effects. To test these hypotheses, we examined how N availability and acute drought affect the levels of hydrogen peroxide (H_2O_2, a key ROS), proline and glutamate in healthy and inoculated shoots of Austrian pine trees. Using in vitro assays, we also evaluated proline's potential to protect *D. sapinea* from direct H_2O_2 damage, the role of the pathogen's antioxidant enzymatic machinery in scavenging H_2O_2 and the ability of *D. sapinea* to utilize various N sources, including proline and glutamate.

Materials and methods

Plant and fungal material

Five-year-old, open pollinated Austrian pine (*P. nigra*) trees from Willoway Nursery (Madison, OH, USA) were potted into 8 l plastic pots using a potting mix of pine bark chips and Com-Til compost (provided by Department of Public Utilities, city of Columbus, OH, USA) in a 3 : 1 ratio (Luchi et al. 2005). An isolate of *D. sapinea* collected from symptomatic Austrian pine cones on The Ohio State Campus (Sherwood and Bonello 2013) was used for all portions of this work.

Greenhouse study

The greenhouse study consisted of a randomized complete block design with three factors: fertilization (fertilized and

unfertilized), watering (watered and droughted) and inoculation (non-inoculated, mock-inoculated and *D. sapinea* inoculated). Trees were conditioned to their assigned nutrient regime for 1 year prior to further experimentation. Fertilized trees received a single administration of 50 g of slow release Florikan (17 : 5 : 8 N : P : K) (Florikan ESA LCC, Sarasota, FL, USA) applied on 15 June 2012, and periodic administrations (every 2 weeks) of 200 ppm of Jack's Professional (20 : 10 : 20 N : P : K) water-soluble fertilizer (JR Peters Inc., Allentown, PA, USA) from 15 June 2012 through 31 October 2012. These application levels are considered high for containerized conifers (Landis et al. 1989). Jack's Professional was always applied to capacity. Unfertilized trees were given no supplemental N, but were watered to capacity at all liquid fertilization events. During the fertilization treatment, all trees were stored outside in an open polyhouse and received regular overhead watering during this time. The polyhouse was covered November 2012 through April 2013, whereupon the polyhouse was again opened and regular overhead watering resumed. To assess the growth responses to the fertilization treatment, stem diameter at 25 cm above the soil line and total plant height were measured at the beginning of the differential fertilization regime, and again on 22 April 2013. Additionally, the total foliar N content was measured from needles collected on 24 April 2013. Needles were collected from each tree, dried at 60 °C for 5 days and ground to a powder in liquid N. For a given fertilization level, equal parts of the ground needles from every eight trees were pooled together, resulting in nine pooled samples for each fertilization level. The N content was measured by the Service Testing and Research Laboratory (Wooster, OH, USA).

On 5 June 2013, trees were transferred from the polyhouse to a greenhouse, and arranged into four blocks, with each block containing three replicates of each unique treatment combination, for a total of 12 replicates for each of the 12 treatment combinations (2 watering types × 2 fertilization levels × 3 inoculation states = 12 treatment combinations), and thus a grand total of 144 trees. At that time, the watering/drought treatment was implemented, with control trees receiving daily watering applied until runoff, and droughted trees receiving no water for the duration of the greenhouse study. To assess the tree response to the differential water regimes, a subsample of 10 trees from each watering treatment was selected and repeatedly sampled at 1, 8, 20, 27, 35 and 56 days post inception of the watering treatment. Two needles per tree were collected, and predawn needle water potential was measured using a Scholander pressure bomb (PMS Instrument Co., Corvallis, OR, USA). During the experiment, greenhouse conditions were monitored, but not controlled, and no additional lighting was supplied. Temperature, relative humidity and irradiance were measured three times daily at 12:15 am (night-time), 8:15 am (morning) and 4:15 pm (daytime). Throughout the experiment, mean nighttime conditions were: 22.0 °C, 70.9% relative humidity and 40.9 W m^{-2}; mean morning conditions were: 26.8 °C, 60.4% relative humidity and 429.4 W m^{-2}; mean daytime conditions were: 26.7 °C, 56.3% relative humidity and 237.4 W m^{-2}.

Inoculations and tissue harvesting Current year shoots were inoculated on 5 July 2013 (Day 35), when the average predawn needle water potential readings for the droughted trees was less than −2.5 MPa (see Figure S1 available as Supplementary Data at *Tree Physiology* Online), which is considered high stress (Blodgett and Stanosz 1997, Blodgett et al. 1997*a*). Inoculations were performed as described in Blodgett et al. (2007), with minor modifications. Briefly, a small, circular wound of ~3 mm in diameter was made on the shoots of the mock-inoculated and inoculated trees using a scalpel, at 5 cm from the tip of the shoot. For inoculated trees, a single 3-mm diameter agar plug taken from the margins of a *D. sapinea* colony, actively growing on potato dextrose agar (PDA, Difco—BD, Franklin Lakes, NJ, USA), was placed mycelium-side down on the wound. In the case of mock-inoculations, sterile, uninoculated PDA was used instead. All inoculations were then wrapped in Parafilm M (Structure Probe, Inc., West Chester, PA, USA) to minimize contamination and desiccation. Non-inoculated control (NIC) trees received no wound or inoculation plugs. Each of the mock-inoculated and inoculated trees was treated twice on separate first year shoots. Inoculations were incubated for 3 weeks, with the watering treatments continuing during the incubation. On 31 July 2013 (Day 56) the shoots were excised from all trees; for the NIC trees, randomly chosen first year shoots were taken. The needles and outer epidermal tissue surrounding the lesions were carefully removed using a scalpel, and lesions were measured by hand using a ruler. After lesion measurement, tissues at the interface of the lesion were excised, immediately frozen in liquid N, ground to a fine powder in liquid N and stored at −80 °C until further processing.

H_2O_2 quantification

H_2O_2 was quantified using a modified Amplex Red method (S. Chakraborty, A. Hill and P. Bonello, unpublished data). For each sample, 100 mg fresh weight (FW) of ground tissue was extracted in 0.05 M sodium phosphate buffer (PB), pH 7.4 for 30 min under continuous shaking at room temperature. Tissue was pelleted via centrifugation (10 min at 20,000 RCF at 4 °C), and the supernatant transferred to a fresh tube. Extracts were immediately quantified using the Amplex Red H_2O_2/Peroxidase Assay kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Amplex Red reactions were carried out in a total volume of 100 µl in black 96-well Microfluor plates (Thermo Fisher Scientific Inc., Waltham, MA, USA). After all components were added to the well, the plates were shaken for 1 min at 250 rpm and then incubated at 30 °C for 30 min in the dark prior to readings. Fluorescence readings were taken using a Victor Wallac (PerkinElmer, Waltham, MA, USA) spectrophotometer

equipped with an AR 560/590 nm filter wheel. H_2O_2 concentrations were determined using a five point standard curve of H_2O_2 included on every plate (in all cases $R^2 > 0.990$) (Thermo Fisher Scientific Inc.).

Amino acid quantification Free AAs were extracted from 50 mg FW of ground plant material for 1 h on a shaker in 500 µl of 0.01 N HCl, pH 7.4. Tissue was pelleted via centrifugation (10 min at 20,000 RCF at 4 °C), and the supernatant transferred to a fresh tube, flash frozen in liquid N and stored at –80 °C until further processing. One hundred µl of the AA extracts were derivatized using the EZ:faast, Free (Physiological) Amino Acid Analysis by GC-MS kit (Phenomenex Torrance, CA, USA), following the manufacturer's instructions. Derivatized AAs were analyzed by gas chromatography-mass spectrometry with a Varian 4000 Saturn GC-MS equipped with a Varian Combi PAL auto-sampler (Varian - Agilent Technologies, Santa Clara, CA, USA). Separation was carried out using a ZB-AAA GC column (10 m, 0.25 mm), included in the EZ:faast kit. Analysis conditions were the following: H_2 (carrier gas) at constant flow of 1.2 ml min^{-1}; injection volume of 2 µl and split injection ratio of 1 : 10; injector temperature of 250 °C. The oven temperature programing started at 110 °C and increased linearly to 160 °C at 15 °C min^{-1}, and then from 160 to 320 °C at 25 °C min^{-1}; total run time was 9.73 min. Ions in the MS were introduced to the ion trap detector employing a negative ion electron ionization, while detection was done using full scan with an m/z range of 65–400. Data acquisition and subsequent processing were performed using MS Workstation software. Amino acids were identified by comparison of their spectra and retention time with those of the 26 standards included in the EZ:faast kit. The absolute amounts of individual AAs were determined by using three-point calibration curves, normalized to the internal standard (norvaline). In all standard curves, three technical replicates were averaged for each concentration. All linear regressions had $R^2 > 0.990$, except for allo-isoleucine ($R^2 = 0.975$), aspartate ($R^2 = 0.984$), glutamate $R^2 = 0.987$, ornithine ($R^2 = 0.989$), tyrosine ($R^2 = 0.978$) and tryptophan ($R^2 = 0.978$). Data are reported as nmoles g^{-1} FW.

In vitro assays

Nitrogen utilization assays *Diplodia sapinea*'s preference for different N sources was examined by growing the fungus on a minimal medium (MM) supplemented with either $NaNO_3$, NH_4NO_3, L-glutamate, L-proline or no N source. MM consisted of 2% agar with 28 mM glucose, 0.9 mM $CaCl_2$ and 2 mM $MgSO_4 \bullet 7H_2O$ in tap water (Griffin et al. 1986) (all reagents from Sigma-Aldrich, St Louis, MO, USA). $NaNO_3$, glutamate and proline were tested at 10, 20 and 30 mM concentrations, while NH_4NO_3 was tested at 5, 10 and 15 mM to keep the total moles of N equal for all compounds. $NaNO_3$, NH_4NO_3 and proline were added, post 0.22 µm filtration, to autoclaved, cooling MM as concentrated stock solutions. Due to its poor solubility in water and its susceptibility to degradation during autoclaving (Davis et al. 1980), glutamate was added directly as a powder to the medium immediately following autoclave sterilization; no contamination was observed in any plates. Eight replicate 8.5 cm plates for each N source by concentration were made, with 25 ml of media per plate. A sterile cellophane disk was placed on top of the solidified agar, and plates were inoculated with a 4-mm mycelial plug taken from the margin of a *D. sapinea* culture actively growing on PDA. The plates were then sealed with Parafilm M and incubated at 24 °C in the dark. After a 5-day growth period, resulting colony diameters were measured along two pre-marked perpendicular directions, and the mycelia scraped from the cellophane and massed.

Proline as ROS protectant Proline's ability to protect *D. sapinea* from damage by H_2O_2 was assessed by exposing mycelia, growing on medium amended with proline, to H_2O_2, and then assessing hyphal tip survival using Evans Blue dye (Sigma), which stains dead or cells with compromised membranes (Chen and Dickman 2005). The fungus was allowed to grow on a thin layer of 2% water agar (WA), with and without 5 or 15 mM proline, that was obtained by solidifying 250 µl of medium into an ~2 cm wide circle on glass slides. Solidified WA was secured to the slide by applying nail polish around its perimeter. Small fungal plugs were excised from stock *D. sapinea* cultures growing on PDA using a scalpel, and placed onto the prepared slides. Slides were placed into Petri dishes and incubated at 24 °C in the dark for 2 days. For each proline by H_2O_2 concentration, eight colonies were prepared with two colonies per slide, for a total of 72 colonies on 36 slides. Following the incubation, slides were submerged in either 0, 5 or 15 mM H_2O_2 in PB saline (PBS), pH 7.4, and incubated for 2 h at room temperature on an orbital shaker (40 rpm, to provide gentle agitation). Slides were then quickly rinsed once in PBS, followed by a 30 min wash in PBS on the shaker to remove the remaining H_2O_2. Following the PBS wash, slides were stained in 0.1% Evans Blue in PBS for 45 min at room temperature on the shaker. Slides were then washed once in PBS quickly, followed by two 15-min washes in PBS with shaking to remove any Evans Blue remaining in the medium. Slides were then examined using a Leica DM750 bright field microscope (Leica Microsystems, Buffalo Grove, IL, USA) to determine hyphal tip survival. The first 100 tips observed were classified as either alive (clear) or dying/dead (stained blue).

H_2O_2 toxicity and enzymatic scavenging assays To determine the effects of H_2O_2 on growth, and the enzymatic H_2O_2 scavenging ability of *D. sapinea*, the fungus was grown on Czapek Dox agar medium (Oxoid, Altrincham, UK), amended with H_2O_2 with final nominal concentrations of 0, 5, 10, 15, 20 and 25 mM H_2O_2; H_2O_2 was added to cooling medium prior to plate pouring.

For each H_2O_2 concentration, 20 replicate 8.5 cm plates were made, each containing 35 ml of medium. Ten plates were used for inoculation with *D. sapinea*, while the remaining 10 were left un-inoculated to assess H_2O_2 breakdown in the medium alone. To quantify the initial H_2O_2 concentration of the plates, agar plugs were removed from non-inoculated plates upon medium solidification. Plugs were immediately flash frozen in liquid N and stored at −80 °C until further processing. All plates then had a sterile cellophane disk added, as described above. Plates in the fungal treatment were inoculated with a 4-mm agar plug taken from the margin of an active *D. sapinea* colony growing on PDA. All plates were sealed with Parafilm M and incubated at 24 °C in the dark for 5 days. Following incubation, non-inoculated plates were again sampled using the same method as above. The diameters of fungal colonies were measured along two pre-marked perpendicular directions. The outer margin of the colonies (an ~1 cm circular band of tissue) was removed from the cellophane using a scalpel. This mycelium was massed, immediately frozen in liquid N, and stored at −80 °C until further processing for catalase and peroxidase activity. The remainder of the colony, with the exclusion of the inoculation plug, was removed from the cellophane and massed to get the total fungal colony FW. Agar plugs in positions corresponding to the outer colony margins (i.e., directly below the excised mycelium) were removed and stored as described above.

H_2O_2 levels in the medium were quantified using the Amplex Red kit. The agar plugs were partially thawed by placing the samples at 4 °C for ~20 min, so that the plugs could be easily crushed by hand using a micropestle. Samples were ground until they were a relatively homogeneous mixture of agar and liquid, and then centrifuged at 4 °C at 15,000 RCF for 5 min to separate the solid agar from the liquid. The supernatant was removed and transferred to a new microcentrifuge tube and immediately analyzed for H_2O_2.

Mycelial catalase and peroxidase activity were specifically selected for assessment, because these enzymes are capable of directly degrading H_2O_2 (the ROS used here), unlike other important ROS scavenging enzymes such superoxide dismutase (SOD), which acts upon superoxide (O_2^-) and free radicals (•OH) (Tainer et al. 1983). The mycelia collected from the outer margin of the colonies were lyophilized at −40 °C for 3 days and then ground to a powder using a micropestle. Proteins were extracted from the powdered mycelia in 500 µl of 0.01 M PB (pH 6.8) for 30 min with constant shaking. Tissue was pelleted by centrifugation at 4 °C at 15,000 RCF for 10 min and the supernatant transferred to a fresh tube. Proteins were precipitated by adding 4.5 ml of ice-cold acetone to the supernatant and incubating the solution for 30 min at −20 °C. Proteins were pelleted via centrifugation at 4 °C at 15,000 RCF for 10 min, after which the supernatant was discarded and the pellet allowed to air dry for 20 min. The proteins were re-suspended in 300 µl of 0.01 M PB (pH 6.8) and quantified as micrograms of BSA protein equivalents per gram FW mycelium using the Coomassie Plus (Bradford) Assay kit (Thermo Fisher Scientific Inc.), following the manufacturer's instructions. Catalase activity was determined spectrophotometrically by measuring the breakdown of H_2O_2 at 240 nm over time using the protocol of Beers and Sizer (1952), with slight modifications, using 2 ml UV-ready disposable cuvettes with 10 µM H_2O_2 in 0.05 M PB, pH 7.4, at room temperature. Five microliters of the protein extract was added to the cuvette, and the solution mixed by manual pipetting for 5 s prior to readings. Readings were taken every 15 s for 3 min per sample. Catalase activity was determined as Δ absorbance at 240 nm min^{-1} mg^{-1} protein. Peroxidase activity was determined using the Amplex Red kit following the manufacturer's instructions. In brief, protein extracts were added to a solution containing a fixed concentration of H_2O_2 and the Amplex Red reagent; plates were shaken, incubated and fluorescence measured as previously described. Peroxidase activity is reported as mU mg^{-1} protein.

Statistical analysis

The effects of fertilization treatment on the increase in stem diameter, plant height and foliar N were determined separately using one-way analysis of variance (ANOVA). To test the effects of watering status, fertilization level and inoculation type on shoot lesion lengths, ROS levels and AA concentrations, we used separate univariate general linear models, including block as a fixed factor. For lesion lengths, mock-inoculations never resulted in any appreciable lesions, thus the factor 'inoculation' was not included in the model. Differences in lesion lengths due to inoculation were determined using Fisher's LSD post-hoc test. Amino acid concentrations required square root transformation in order to meet the model assumptions. Outliers (±3 SD) were removed to improve data normality and homoscedasticity; no more than two cases per treatment combination were removed, and no trimming was required for most compounds. Differences in ROS and AA concentrations due to inoculation type within a given water by fertilization level were determined using Fisher's LSD test.

For the in vitro experiment with MM, differences in colony diameter and FW mass were determined using separate one-way ANOVAs coupled with post-hoc Fisher's LSD tests. For the hyphal tip survival study, we first tested the effects of media and H_2O_2 treatment on the percentage of hyphal tip survival with a general linear model. Since both factors had a significant effect (Media $P = 0.005$; H_2O_2 treatment = <0.001), but the interaction was not significant ($P = 0.189$), we split the analyses to test only for the effect of proline concentration within an H_2O_2 treatment using one-way ANOVA. The proline concentrations were differentiated using Fisher's LSD test. The effects of H_2O_2 on colony diameter, colony mass, catalase and peroxidase activities were determined using separate one-way ANOVAs, where H_2O_2 concentration was the treatment factor. Differences in colony diameter, masses and enzyme activities between the

different H_2O_2 concentrations were determined using Fisher's LSD test. Differences in the detected H_2O_2 from the agar plugs on Day 0 and 5 were determined using separate one-way ANOVAs followed by Fisher's LSD tests to determine differences in the effects of the applied concentrations.

For all general linear models, non-significant interactions (where $P > 0.10$) were removed, and the model re-run. All statistical analyses were performed with SPSS (v. 21, IBM, Armonk, NY, USA), using $\alpha = 0.05$.

Results

Greenhouse study

Fertilization and drought establishment Fertilization significantly increased stem diameter, plant height and foliar N content (see Table S1 available as Supplementary Data at *Tree Physiology* Online). Drought treatment continually decreased needle water potentials, reaching a mean value of −2.56 MPa on Day 35 and −2.81 MPa on Day 56, while the watered trees remained around −1.0 MPa for the experiment's duration (see Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Shoot lesion lengths For the inoculated trees, drought treatment significantly increased shoot lesion lengths ($F_{1,47} = 18.56$; $P = 0.001$), but fertility had no effect ($F_{1,47} = 0.59$; $P = 0.447$) (Figure 1). No effect of watering or fertilization was observed for the mock-inoculated trees (data not shown).

Shoot H_2O_2 quantification Watering status, fertilization level and inoculation type all significantly affected shoot H_2O_2 concentrations, with drought significantly increasing shoot H_2O_2 levels and fertilization decreasing H_2O_2 levels (Table 1, Figure 2). Fungal inoculation decreased H_2O_2 for all watering by fertilization treatment combinations (Figure 2).

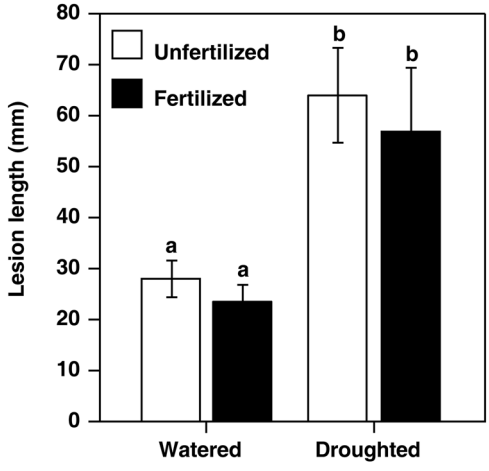

Figure 1. Drought increased shoot lesion lengths (mean ± SE), measured 3 weeks after inoculation with *Diplodia sapinea*, while fertilization had no effect. Different letters represent significant differences, according to Fisher's LSD test at $\alpha = 0.05$.

Amino acid profiling Mean concentrations for all AAs are reported in Tables S2–S4 available as Supplementary Data at *Tree Physiology* Online. The results of the general linear model for all AAs except proline and glutamate are reported in Table S5 available as Supplementary Data at *Tree Physiology* Online. Significant main effects of watering, fertilization and inoculation were detected for the majority of the AA analyzed (see Table S5 available as Supplementary Data at *Tree Physiology* Online). Significant interactions between watering status and fertilization level, and watering status and

Table 1. ANOVA results of the general linear models testing effects of watering, fertilization and inoculation treatments on shoot H_2O_2, proline and glutamate concentrations 3 weeks after inoculation. Non-significant interactions removed from models. *P*-values <0.05 are highlighted in bold.

Compound	Source of variation	df	MS	*F*-value	*P*-value
H_2O_2	Watering status	1	62	8.86	**0.003**
	Fertilization level	1	60	8.47	**0.004**
	Inoculation type	2	200	28.44	**<0.001**
Proline	Watering status	1	8614	290.4	**<0.001**
	Fertilization level	1	993	33.5	**<0.001**
	Inoculation type	2	591	19.9	**<0.001**
	Watering status × fertilization level	1	889	30.0	**<0.001**
	Watering status × inoculation type	2	145	4.9	**0.009**
Glutamate	Watering status	1	2037	49.5	**<0.001**
	Fertilization level	1	257	6.3	0.140
	Inoculation type	2	85	2.1	0.129
	Watering status × inoculation type	2	558	13.6	**<0.001**

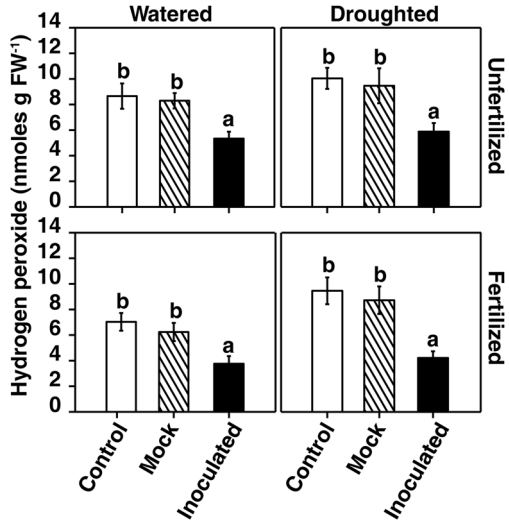

Figure 2. Effects of drought and fertilization treatments on H_2O_2 levels (mean ± SE) of Austrian pine shoots 3 weeks after inoculation with *Diplodia sapinea*. Different letters represent significant differences between inoculation types within a given watering status by fertilization level combination, according to Fisher's LSD test at $\alpha = 0.05$.

inoculation type were frequently detected as well (see Table S5 available as Supplementary Data at *Tree Physiology* Online). Differences in concentrations due to inoculation type were found for all AAs (not including proline and glutamate), as shown in Table S6 available as Supplementary Data at *Tree Physiology* Online.

Watering status, fertilization level and inoculation type all significantly impacted proline concentration, with significant watering by fertilization and watering by inoculation interactions detected (Table 1). Drought treatment, fungal inoculation and fertilization significantly increased shoot proline levels (Figure 3, Table S2–S4 available as Supplementary Data at *Tree Physiology* Online). Post-hoc analyses on the inoculation type within a given watering and fertilization level show that inoculated plants had higher levels of proline in all watering and fertilization combinations compared with the NIC and mock-inoculated trees (Figure 3).

Watering status, fertilization level and inoculation type all significantly affected glutamate levels as well, with a significant watering by inoculation type interaction and a significant main effect of fertilization (Table 1). Depending on the fertilization level, glutamate was either not different or slightly higher in the watered, inoculated trees, but for both fertilization levels in droughted trees the inoculated plants always had lower glutamate levels than the NIC and mock-inoculated trees (Figure 4, Table S2–S4 available as Supplementary Data at *Tree Physiology* Online).

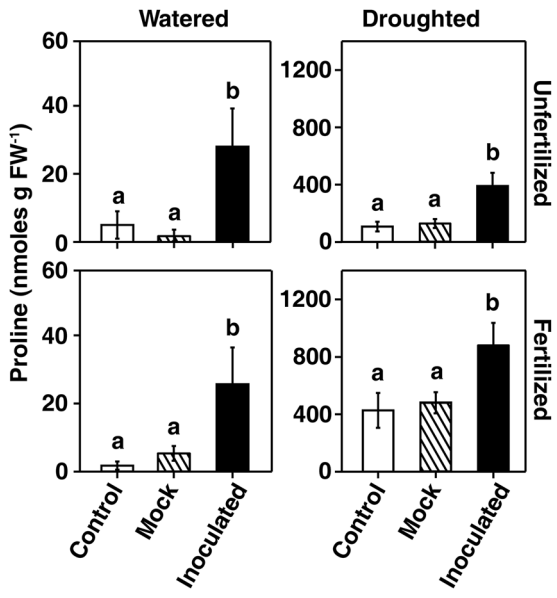

Figure 3. Effects of drought and fertilization treatments on proline levels (mean ± SE) of Austrian pine shoots 3 weeks after inoculation with *Diplodia sapinea*. Note the different scale between watered and droughted samples. Different letters represent significant differences between inoculation types within a given watering status by fertilization level, according to Fisher's LSD test at $\alpha = 0.05$.

In vitro studies

Nitrogen utilization assays Proline was the most preferred N source out of the four tested, resulting in the largest mean colony diameters and highest mycelial FW, and all concentrations of proline tested resulted in significantly greater masses and diameters compared with the N-free MM control, except for colonies on 10 mM proline, which showed no significant increase in diameter, but did increase in FW (Figure 5). NH_4NO_3 was also a preferred N source, always resulting in higher mycelial masses and larger diameters than those of the MM controls and having larger diameters at 5 mM than the 10 mM proline colonies (concentrations refer to NH_4NO_3; therefore, total N is double). However, FW were lower in the 10 and 15 mM plates compared with the 5 mM plates, indicating potential mild toxicity at the higher concentrations, although there was no decrease in diameters at these concentrations. $NaNO_3$ decreased colony diameters at all concentrations and had either no effect on FW (when at 10 and 20 mM) or increased FW (30 mM) compared with the MM controls. The diameters of $NaNO_3$-fed colonies were always smaller than those of colonies fed NH_4NO_3 and proline, and their FW were always lower than those growing in NH_4NO_3 and proline, except for 10 mM proline colonies, which were similar to the 30 mM $NaNO_3$ colonies. Glutamate reduced growth at all concentrations, resulting in smaller colonies with very low masses. In all cases, the FW of the glutamate-fed colonies were lower than those of all the other N sources tested and the MM. Diameters of the glutamate-fed colonies were either equivalent to or smaller than $NaNO_3$- and MM-fed colonies, with the 30 mM glutamate colonies having the smallest diameters of any N source.

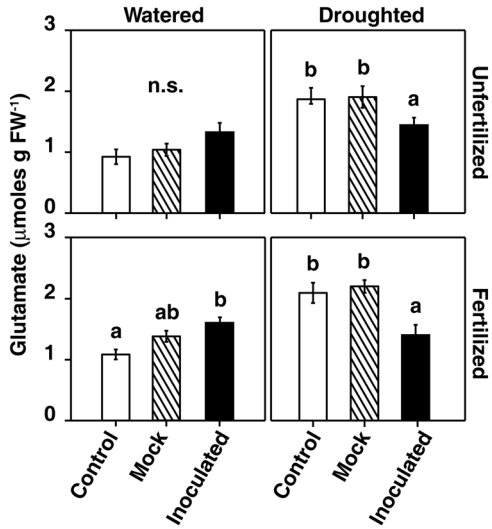

Figure 4. Effects of drought and fertilization treatments on glutamate levels (mean ± SE) of Austrian pine shoots 3 weeks after inoculation with *Diplodia sapinea*. Different letters represent significant differences between inoculation types within a given watering status by fertilization level, according to Fisher's LSD test at $\alpha = 0.05$. n.s., not significant.

Proline as an ROS protectant In the hyphal tip survival assay, both the media and the H_2O_2 treatment affected the percentage of tip survival (Table 2). However, there was no significant effect of proline concentration on tip survival for the 0 mM H_2O_2 ($F_{2,23} = 0.17$; $P = 0.846$) or the 5 mM H_2O_2 ($F_{2,23} = 1.26$; $P = 0.305$) treated colonies. Proline increased tip survival in the 15 mM H_2O_2 treatment ($F_{2,23} = 10.09$; $P < 0.001$), with the 5 and 15 mM proline colonies having higher tip survival percentages than the WA, but with no difference between 5 and 15 mM proline treatments (see Figure S2 available as Supplementary Data at *Tree Physiology* Online).

H_2O_2 toxicity and enzymatic scavenging assays H_2O_2 reduced *D. sapinea* colony diameter, following a dose–response pattern (Figure 6). Colony FW also decreased at >15 mM H_2O_2 concentrations, but there were no differences in mass within the 0–10 mM concentrations or the 15–25 mM concentrations. The mycelia from the outer margins of the colonies displayed increasing catalase and peroxidase activities with increasing H_2O_2 concentrations (Figure 7). Catalase activity increased in near-linear fashion above the 0 mM H_2O_2 basal level in the 10–25 mM H_2O_2 plates, with the highest activity in the 25 mM H_2O_2 plates, which was approximately five times the activity of the 0 mM H_2O_2 plates. Peroxidase activity increased significantly only at 25 mM H_2O_2, but there was a gradual, near-linear increase in activity with increasing H_2O_2.

Measured H_2O_2 concentrations in non-inoculated plates at Day 0 were ~5–7% of the applied amount, indicating a rapid degradation of H_2O_2 upon addition to the media, but a clear concentration gradient was still present with significant differences detected between all concentrations (see Table S7

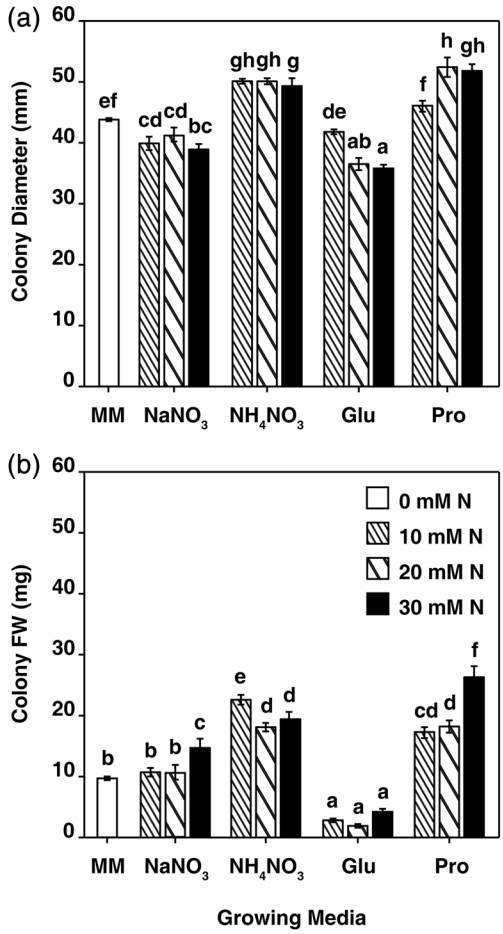

Figure 5. Effects of different N sources on (a) *Diplodia sapinea* colony diameters (mean ± SE) and (b) FW masses (mean ± SE) after 5 days growth. Different media correspond to N-lacking MM, either alone or amended with different concentrations of sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3), glutamate (Glu) or proline (Pro). N molarity is equal for all compounds within a given N concentration. Different letters represent significant differences, according to Fisher's LSD test at $\alpha = 0.05$.

Table 2. ANOVA results of the general linear model testing effects of media type and H_2O_2 on hyphal tip survival. *P*-values <0.05 are highlighted in bold.

Source of variation	df	MS	*F*-value	*P*-value
Media	2	717	5.83	**0.005**
H_2O_2 treatment	2	1991	16.20	**<0.001**
Media × H_2O_2 treatment	4	195	1.59	0.189

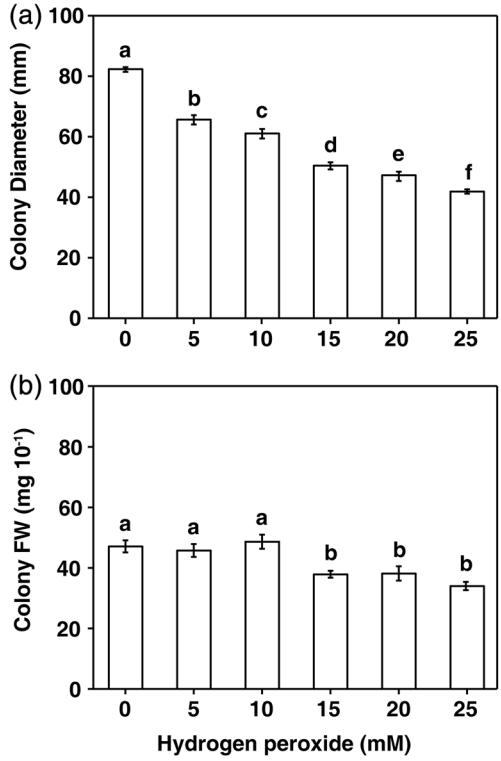

Figure 6. Hydrogen peroxide added to Czapek Dox agar reduced (a) *Diplodia sapinea* colony diameters (mean ± SE) and (b) FW masses of *D. sapinea* colonies (mean ± SE) grown for 5 days on Czapek Dox agar amended with different H_2O_2 concentrations. Within either colony diameters or colony FW masses, respectively, different letters represent significant differences, according to Fisher's LSD test at $\alpha = 0.05$.

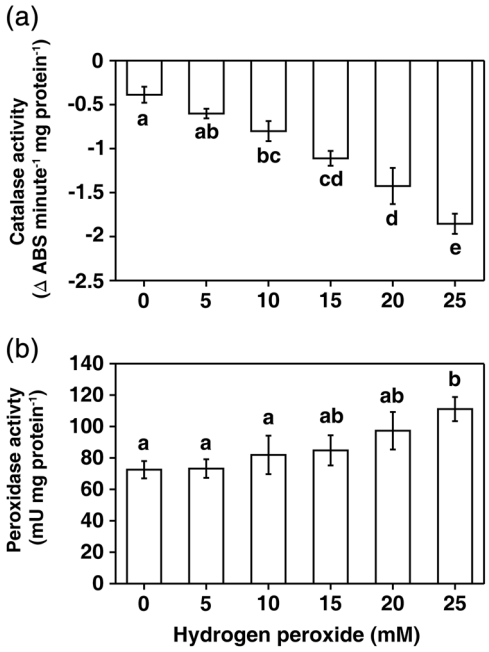

Figure 7. Increasing catalase (a) and peroxidase (b) activities (mean ± SE) of the mycelia from *Diplodia sapinea* colonies increased when grown for 5 days on Czapek Dox agar amended with different H_2O_2 concentrations. Catalase activity was determined as the loss of H_2O_2 overtime (measured at 240 nm), therefore lower values correspond to greater catalase activity. Different letters represent significant differences, according to Fisher's LSD test at $\alpha = 0.05$. ABS, absorbance.

available as Supplementary Data at *Tree Physiology* Online). Comparisons of the detected H_2O_2 at Day 5 revealed there were still significant differences across the applied H_2O_2 for the non-inoculated plates (see Figure S3 available as Supplementary Data at *Tree Physiology* Online). However, the inoculated plates showed large decreases in measured H_2O_2, with no significant differences between any of the applied H_2O_2 treatments, and all treatments being equivalent to the 0 mM H_2O_2 of the Day 5 non-inoculated plates (see Figure S3 available as Supplementary Data at *Tree Physiology* Online).

Discussion

As expected, trees experiencing water stress were more susceptible to *D. sapinea* infection, as evidenced by longer shoot lesions, with the droughted trees having lesions nearly twice as long as those of the watered plants. This marked increase in susceptibility is well documented with this pathogen (Blodgett et al. 1997*a*, 1997*b*, Paoletti et al. 2001, Stanosz et al. 2001, Desprez-Loustau et al. 2006). Drought treatment also triggered the accumulation of all AAs and H_2O_2 in the shoots. Inoculated trees had higher levels of proline compared with the NIC and mock-inoculated trees for both watered and droughted trees. Proline was the only AA to have further increased beyond the levels in the NIC and mock-inoculated trees in the droughted trees as a result of fungal attack (Figure 3 and Tables S4 and S6 available as Supplementary Data at *Tree Physiology* Online). The accumulation of proline during infection is not uncommon (Hare and Cress 1997), but its accumulation has been associated with resistance to pathogens via the P5C-proline cycle-mediated HR (Cecchini et al. 2011, Senthil-Kumar and Mysore 2012, Monteoliva et al. 2014), and to our knowledge has not been associated with susceptibility phenotypes. Therefore, higher levels of proline were unexpected in association with greater susceptibility. We attribute this outcome to *D. sapinea*'s necrotrophic lifestyle, as necrotrophs can hijack plant cellular processes, like HR, to facilitate their own infection process (Govrin and Levine 2000, van Kan 2006, Hammond-Kosack and Rudd 2008, Laluk and Mengiste 2010).

Seifi et al. (2013*b*) described the P5C-proline cycle as part of an 'evasion' strategy, in which PCD (which is used to seal off invading pathogens) is coupled with the active mobilization of nutrients away from the infection site to starve pathogens. Opposite and alternative to an evasion strategy is 'endurance', whereby cell viability is maintained and resources are gathered near the infection site as energy to combat infection (Seifi et al. 2013*b*). Evasion is predicted to be successful against biotrophic pathogens, which feed on living host tissue, while endurance would be successful against necrotrophic pathogens, which feed on killed host material. Therefore, the P5C-proline cycle would be beneficial to necrotrophs because it is an evasion response.

The accumulation of certain AA (e.g., proline) during infection suggests that similar shifts in AA metabolism, occurring during water stress, may predispose the plant to certain pathogen classes (i.e., biotrophs or necrotrophs) by shifting host physiology to either an evasion or an endurance-like state. To our knowledge, drought and other abiotic stress responses have not been classified in this manner, but the work of Seifi et al. (2013*b*) provides a useful framework for predicting the outcomes between stressed plants and pathogens. The P5C-proline cycle would seem a useful target for manipulation by *D. sapinea* during drought, because proline synthesis is already amplified. The observed increase in proline, coupled with the decrease in glutamate (proline's primary precursor), supports the notion that proline synthesis, but not its complete catabolism, occur during drought and infection, which suggests induction of the P5C-proline cycle. The cycle could also serve as a general target for manipulation by *D. sapinea* during infection of non-droughted plants, as proline levels increased in the inoculated, watered trees as well. Further genetic studies are needed to confirm whether this hypothesis has merit.

Contrary to our initial thoughts, fertilization had no effect on disease severity in either the droughted or watered trees. However, fertilization did significantly affect the AA content of the shoots, with the fertilized trees typically having greater AA accumulation in the drought treatment compared with the unfertilized, droughted trees. This was in agreement with our hypothesis that

higher N availability would allow for greater synthesis and accumulation of AA. Differences in AA levels based on N fertilization have been observed in other plant systems (Näsholm and McDonald 1990, Påhlsson 1992, Ohlson et al. 1995) including pine (Kim et al. 1987, Edfast et al. 1990, Näsholm and Ericsson 1990), but these studies focused only on constitutive AA levels. The combined effects of fertilization and drought are comparatively less studied than the individual factors alone, particularly in regards to the molecular composition of plant tissues, but in a few studies on tree species, fertilization had little effect on drought-associated AA accumulation (Parker and Patton 1975, Green et al. 1994), which is contrary to our results. Nonetheless, the watering by fertilization interaction observed for most AA, and the frequently observed main effect of fertilization level, demonstrates that the plant's N supply will at least partially determine plant AA composition and differential accumulation during stress events.

The ramifications of fertilization-dependent AA accumulation on plant resistance to pathogens seem minimal in this pathosystem, but this could be because other defense mechanisms are enhanced by the fertilization treatment. The greater AA accumulation in the fertilized plants may be increasing the susceptibility of the shoots via higher proline levels as hypothesized, but this susceptibility could be offset by a greater investment in the other defensive mechanisms, like phenolics and defensive proteins the amounts of which are all known to be affected by the N availability. For example, Barto et al. (2008) demonstrated that fertilization increases the activities of several defensive proteins in the needles and stems of Austrian pines, while in a related study, Wallis et al. (2011) found that total phenolics and certain individual compounds increase with fertilization. Another possible explanation for the observed lack of N fertilization effects on resistance may reside in the absolute differences in levels between fertilized and unfertilized trees, which, while statistically significant, may be negligible in terms of direct benefits to the pathogen. A more quantitative method of fertilization (e.g., Wallis et al. 2008, 2011) may be more sensitive for detecting fertilization effects on resistance due to changes in AA or other responses.

The increase in shoot H_2O_2 levels in the droughted trees was expected, indicating that the trees were experiencing oxidative stress. Fertilization also affected shoot H_2O_2 levels, with the unfertilized trees having greater amounts on average. This may have occurred for a variety of reasons. For instance, the fertilized plants may have had more proteinaceous antioxidants and scavenging systems due to higher N levels in the tissues. Proline levels may also explain the effect of fertilization on shoot H_2O_2 levels; the fertilized plants accumulated more proline than the unfertilized plants during drought, and if proline is scavenging H_2O_2 directly (Matysik et al. 2002), then lower shoot H_2O_2 would be expected in the fertilized plants. For shoot H_2O_2, mock inoculated plants mirrored the NIC plants in their respective watering statuses and fertilization levels, but fungal inoculated plants always had lower shoot H_2O_2 amounts. This suggests that H_2O_2 removal may be a general infection strategy employed by *D. sapinea*, regardless of water stress or N status. Reactive oxygen species are damaging to invading pathogens, so their removal should facilitate infection and fungal growth. This was seen in the in vitro studies, where H_2O_2 reduced fungal growth. Hydrogen peroxide is also used by plants for defensive signaling (Low and Merida 1996, Orozco-Cárdenas et al. 2001, Neill et al. 2002), so its removal during infection may improve pathogenicity by thwarting defensive responses, a strategy observed in several plant pathosystems (Mellersh et al. 2002, Huang et al. 2011, Chung 2012).

In their review of mechanisms of survival used by necrotrophic fungi during plant HR and ROS bursts, Mayer et al. (2001) highlight several examples where the pathogens use ROS scavenging enzymes to detoxify their surroundings. Best studied are SOD, catalase and peroxidases. For this study, catalase and peroxidase were chosen because they both act directly upon H_2O_2, which was the ROS used here, while SOD does not. *Diplodia sapinea* peroxidase and catalase activities increased with exposure to H_2O_2. Catalase was more responsive than peroxidase, suggesting that catalase is the principal means of removing exogenous H_2O_2 by *D. sapinea*. This is in general agreement with other fungal species (Mayer et al. 2001), though not all (Gil-ad and Mayer 1999). The assay used here utilized horseradish peroxidase as the standard peroxidase and Amplex Red as its substrate. Horseradish peroxidase is a Class III peroxidase (Conesa et al. 2002), and is only one of several types of peroxidases present in fungi (Welinder 1992, Conesa et al. 2002). Peroxidases can utilize a variety of substrates as electron donors, but can be highly specific depending on the enzyme's active site. The ability, or lack thereof, of *D. sapinea* peroxidases to utilize Amplex Red as a substrate is unknown, and so the peroxidase activity observed here may not completely reflect that of the mycelial extracts. The breakdown of H_2O_2 by *Botrytis cinerea* is largely via peroxidase activity (Gil-ad and Mayer 1999, Gil-ad et al. 2000), and so peroxidases may still be an important means of H_2O_2 removal for *D. sapinea*. Regardless of the mechanism of degradation, *D. sapinea* was able to significantly reduce H_2O_2 levels in the agar to near zero levels. The initial (Day 0), measured H_2O_2 concentrations were only ~5–7% of the applied amount, and so most of the H_2O_2 is lost immediately upon addition to the cooling medium. Even with this immediate loss of the majority of H_2O_2, there was a decrease in colony diameters in a concentration-dependent manner, indicating that *D. sapinea* is rather sensitive to small changes in H_2O_2. This supports the notion that removal of H_2O_2 is important for successful infection by *D. sapinea*. In addition to its own enzymatic removal of H_2O_2, host proline may be scavenging H_2O_2, further protecting *D. sapinea* from damage. A protective role for proline was observed in the hyphal tip survival assay, but with this assay it is

not possible to determine whether proline was directly removing H_2O_2 or if proline was being consumed and simply providing energy and nutrients for endogenous protection mechanisms (e.g., catalase). In either case, we suggest that in planta free proline provides protection against damage from H_2O_2 for *D. sapinea*.

Pathogens derive their required N from the host, and plant free and protein-bound AAs are commonly consumed by pathogenic fungi, to meet their N demands (Solomon et al. 2003). This may be especially true for drought-stressed plants, which accumulate AA and other substrates above normal levels. These elevated levels provide an added N benefit for the fungi, as total AA content can reach mM concentrations in some droughted plants (Solomon et al. 2003). Furthermore, drought may trigger the accumulation of certain AA that would otherwise be at negligible levels or entirely absent in healthy plants. This trend was observed for allo-isoleucine, glycine, isoleucine, leucine, lysine, ornithine, phenylalanine, proline, tyrosine and valine (see Table S2 available as Supplementary Data at *Tree Physiology* Online). The availability or increased abundance of these AA in the droughted trees may contribute to their higher levels of disease, because there would be a greater amount and more varied types of N available to *D. sapinea*. The accumulation of proline in droughted trees from near zero levels in the watered controls may be of particular importance for *D. sapinea* nutrition, because proline was clearly a good substrate for this fungus. Furthermore, plant anabolism of proline from glutamate would simultaneously provide a preferred N source and reduce levels of an apparently toxic metabolite, although the observed effects of glutamate may have resulted from the acidification of the MM rather than true toxicity. The preference for other AA and other accumulating metabolites should be examined to get a clearer understanding of what may be contributing to pathogen nutrition during drought.

The genetic and molecular tools available for this pathosystem are presently very limited, making in planta investigations difficult, if not impossible. Thus, in vitro testing of the relevant metabolites was the only avenue to link biochemical changes in the plant with effects on the pathogen. However, the conclusions reached in this way come with caveats. For example, the concentrations of AA and H_2O_2 used for the in vitro assays were higher than those detected in the shoots. The reported compound concentrations are averages for the tissue as a whole, since the shoot tissue was homogenized prior to compound quantification. However, plant tissues and cells are heterogeneous, so the concentration of any given metabolite can be highly variable from cell to cell. For instance, H_2O_2 distribution varied at different locations within the cell during defense responses (Thordal-Christensen et al. 1997), while AA concentrations can change in different parts of a plant cell (Wagner 1979). Similar localization of metabolites may have occurred in this study, in which case the local in planta concentrations encountered by the fungus, e.g., at the hyphal tip interface, could be significantly higher or lower than those reported here. Histological methods could prove useful for examining these phenomena, for example by visualizing the amount of H_2O_2 surrounding an invading hypha.

Taken together, our data provide insights into the mechanisms of drought-induced susceptibility of Austrian pine to *D. sapinea*. We propose that *D. sapinea* activates the P5C-proline cycle to facilitate host cell death and that this cycle can become hyperactive during periods of drought, leading to greater disease severity. To protect itself from damaging ROS formed in response to drought, and those potentially arising from the P5C-proline cycle, *D. sapinea* utilizes ROS-scavenging enzymes like catalases and peroxidases. Additional protection from ROS is also provided by the scavenging activity of proline, which accumulates in response to both drought and infection. Furthermore, this removal of ROS may diminish host defensive signaling (Bolwell and Daudi 2009, Torres 2010), thereby limiting the tissue's defensive potential and creating a conducive environment for pathogens. Lastly, the accumulating proline acts as a preferential N source for the fungus. All these factors together promote enhanced virulence and the greater disease susceptibility observed in droughted Austrian pines (Figure 8).

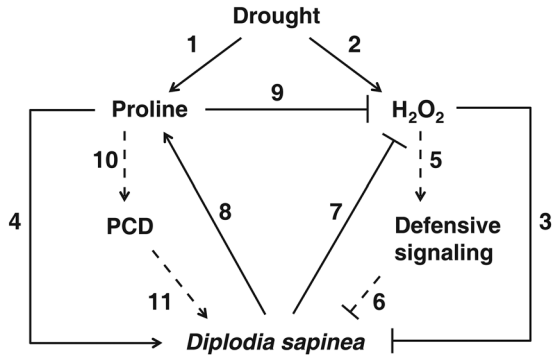

Figure 8. Proposed model for drought-induced Austrian pine susceptibility to the necrotrophic pathogen *Diplodia sapinea*. Drought induces the accumulation of free proline (1) and H_2O_2 (2) in the host, resulting in opposite effects on necrotroph performance, with H_2O_2 being directly toxic to *D. sapinea* (3) and proline being beneficial to the pathogen because it acts as a nutrient source (4). H_2O_2 is also involved in host defensive signaling (5) and the triggering of host defense responses, which are detrimental to the pathogen (6). In response, the invading pathogen detoxifies H_2O_2 using ROS scavenging enzymes like catalase (7), thus limiting its toxicity and interfering with defensive signaling. Proline level further increases in response to pathogenic attack (8), and in addition to serving as a nutrient source, it can scavenge H_2O_2 (9), thereby reducing direct oxidative damage to the pathogen (3) as well as plant defensive signaling (5 and 6). Furthermore, *D. sapinea*'s enhancement of the plant's proline metabolism can trigger PCD (10) via the proline/pyrroline-5-carboxylase cycle, promoting the pathogen's necrotrophic lifestyle of feeding on killed host material (11). Symbols: solid lines represent hypotheses drawn from this study, while dashed lines are hypotheses based on relevant literature; lines ending with arrowheads represent positive effects (e.g., promoting or triggering), while lines terminating with a cross line represent negative effects (e.g., scavenging/removal, interference, inhibition or toxicity).

Multifactorial studies are considered vital for a better understanding of the impacts of a changing environment on plant disease (Mittler 2006). By characterizing the molecular and physiological responses of plants to various stresses in terms of endurance or evasion strategies (Seifi et al. 2013*b*), we can begin to predict how the changing climate will regulate forest health in the future. This will be of particular importance as we continue to develop plant varieties tolerant to drought and other abiotic stress, as the tolerance traits selected for may result in changes in plant physiology that compromise defenses against pathogens and other biotic stressors.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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