

Early plant defence against insect attack: involvement of reactive oxygen species in plant responses to insect egg deposition

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Abstract

Main conclusion *Pinus sylvestris* responds to insect egg deposition by ROS accumulation linked with reduced activity of the ROS scavenger catalase. Egg mortality in needles with hypersensitive response (HR)-like symptoms is enhanced.

Aggressive reactive oxygen species (ROS) play an important role in plant defence against biotic stressors, including herbivorous insects. Plants may even generate ROS in response to insect eggs, thus effectively fighting against future larval herbivory. However, so far nothing is known on how ROS-mediated plant defence against insect eggs is enzymatically regulated. Neither do we know how insects cope with egg-induced plant ROS. We addressed these gaps of knowledge by studying the activities of ROS-related enzymes in *Pinus sylvestris* deposited with eggs of the herbivorous sawfly *Diprion pini*. This species cuts a slit into pine needles and inserts its eggs into the needle tissue. About a quarter of egg-deposited needles show chlorotic tissue at the oviposition sites, indicating hypersensitive response-like direct defence responses resulting in reduced larval hatching from eggs. Hydrogen peroxide and peroxidase sensitive staining of sections of egg-deposited pine needles revealed the presence of hydrogen peroxide and peroxidase activity in needle tissue close to the eggs. Activity of ROS-producing NADPH-oxidase did not

increase after egg deposition. However, the activity of the ROS-detoxifying enzyme catalase decreased after egg deposition and ovipositional wounding of needles. These results show that local ROS accumulation at the oviposition site is not caused by increased NADPH-oxidase activity, but reduced activity of pine needle catalase may contribute to it. However, our data suggest that pine sawflies can counteract the egg deposition-induced hydrogen peroxide accumulation in pine needles by high catalase activity in their oviduct secretion which is released with the eggs into pine tissue.

Keywords Catalase · Direct defence · Hydrogen peroxide · Hypersensitive like response (HR) · Oviposition · *Pinus*

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
HR	Hypersensitive response
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Reactive oxygen species (ROS) play an important role in plant responses to abiotic and biotic stressors (O'Brien et al. 2012). ROS are generated under both stressful and stress-free conditions. Plant metabolism steadily leads to partial reduction of oxygen which results in formation of, for example, the superoxide radical, hydrogen peroxide or hydroxyl radicals as metabolic byproducts. Under normal growth conditions, the level of cell toxic ROS is kept in homeostasis by an effective antioxidant system. However, enhanced ROS concentrations are detectable under stressful

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conditions (Mittler 2002; Apel and Hirt 2004). High ROS concentrations may arise due to the imbalances of metabolic pathways, to increased activity of ROS-generating enzymes, such as NADPH oxidase, and/or to downregulation of scavenging/antioxidant systems (Mittler et al. 2004). Under stressful conditions, ROS can serve as signals that mediate stress responses which induce defensive transcriptional changes that result—in concert with direct ROS effects—in obvious cellular changes, including reinforcement of the plant cell wall by oxidative cross-linking, lignification and cell death (Lamb and Dixon 1997; Mittler et al. 2011; Baxter et al. 2014; Barros et al. 2015; Reczek and Chandel 2015). Stress-induced cell death is a well-known plant response to phytopathogens. The programmed cell death isolates and traps invading (hemi)biotrophic pathogens which require living plant tissue (Reape et al. 2008; Bozhkov and Lam 2011). These plant reactions have also been referred to as hypersensitive response (HR) to phytopathogens (Mur et al. 2008; Coll et al. 2011). ROS are known as positive regulators of HR in many, albeit not all plant–phytopathogen interactions (Torres 2010).

HR-like symptoms have also been observed in plant responses to egg depositions by herbivorous insects (Hilker and Fatouros 2015, 2016). A HR-like plant response to eggs laid onto leaves may lead to the formation of necrotic plant tissue at the site of oviposition. Insect eggs on necrotic leaf tissue quickly desiccate or fall off from the necrotic, dry leaf tissue. Thus, a plant can effectively defend against herbivorous insects in a very early stage of infestation. HR-like responses to insect eggs have been shown in several brassicaceous plants deposited with butterfly eggs (Little et al. 2007; Fatouros et al. 2012, 2015) but also in other herbaceous plants (Poaceae, Fabaceae, Apocynaceae, Solanaceae) in response to the eggs laid by planthoppers, beetles or moths (Hilker and Fatouros 2016). Histochemical staining of *Arabidopsis thaliana* leaves treated with the extracts of butterfly eggs revealed production of ROS at the site of treatment (Little et al. 2007; Gouhier-Darimont et al. 2013; Reymond 2013).

While enzymes involved in HR to phytopathogen infection have intensively been studied (e.g. Almagro et al. 2009; Coll et al. 2011), no knowledge is available on the activities of enzymes involved in HR-like plant responses to insect eggs.

ROS generation in response to phytopathogens requires several enzymes, including the most intensively studied enzyme NADPH oxidase (Morel and Dangl 1997; Meng and Zhang 2013). The superoxide radicals generated by e.g. NADPH oxidase are dismutated to hydrogen peroxide by superoxide dismutase (SOD). When hydrogen peroxide meets iron or copper ions, hydroxyl radicals are formed (Navrot et al. 2007). Plants can control once generated ROS by a set of antioxidant enzymes, including catalase (CAT) which removes hydrogen peroxide by converting it into water and

oxygen, and several peroxidases which can reduce hydrogen peroxide as well. Among the peroxidases, ascorbate peroxidase (APX) is considered a key player in plants (Caverzan et al. 2012) using ascorbic acid as an electron donor. Other known peroxidases catalyse this reaction by reducing agents with a thiol group (glutathion) or hydroxyl groups (phenolics) as electron donor molecules (Passardi et al. 2005).

Here, we addressed the question how insect egg deposition affects ROS-generating and scavenging enzyme activities of a plant. We focused on the enzymes mentioned above that are known to be involved in responses to phytopathogens: NADPH oxidase as ROS-generating enzyme, SOD as a ROS converting enzyme, and CAT and APX as ROS-scavenging enzymes. We used *Pinus sylvestris* (Scots pine) deposited with eggs of the herbivorous insect *Diprion pini* (pine sawfly) for our studies.

We chose this plant–insect system because we observed HR-like symptoms (brownish tissue) in pine needles with sawfly egg depositions (Fig. 1), indicating that ROS enzyme activities are affected by the egg deposition. In addition, no gymnosperm species has so far been studied with respect to insect egg-induced HR-like responses and the role of enzymes involved in these responses. Furthermore, solid knowledge is available that *P. sylvestris* needles can respond to *D. pini* egg deposition. The needles change their pattern of emitted terpenoids in response to sawfly egg deposition, and the egg-induced terpene odour attracts egg parasitoids, thus serving pine anti-herbivore defence (Hilker et al. 2002a; Mumm et al. 2003). The egg-induced terpene odour attracting the parasitoids is not due to the ovipositional wounding which a sawfly inflicts to a pine needle when laying eggs. The sawfly slits a pine needle longitudinally with its ovipositor and inserts the eggs in a row into the slit needle (Fig. 1a). The parasitoid-attracting odour emitted by Scots pine in response to sawfly egg deposition is elicited by the protein-rich oviduct secretion which a sawfly releases with its eggs and which is in tight contact with the internal needle tissue (Hilker et al. 2005). Scots pine does not only change its terpene pattern in response to sawfly egg deposition, but also its expression of genes encoding terpene synthases (Köpke et al. 2008, 2010). Furthermore, sawfly egg deposition has been suggested to be taken by pine as a warning signal of impending larval herbivory since egg-deposited pine twigs increase their defence efficiency against hatching larvae (Beyaert et al. 2012).

Since *D. pini* is highly specialised on *Pinus* species as host plants, we also asked whether it has developed counter-adaptations to putative egg-induced plant ROS. Hence, we studied the question whether the eggs or secretions released with the eggs exhibit ROS-scavenging CAT activity which would protect the eggs from plant produced ROS.

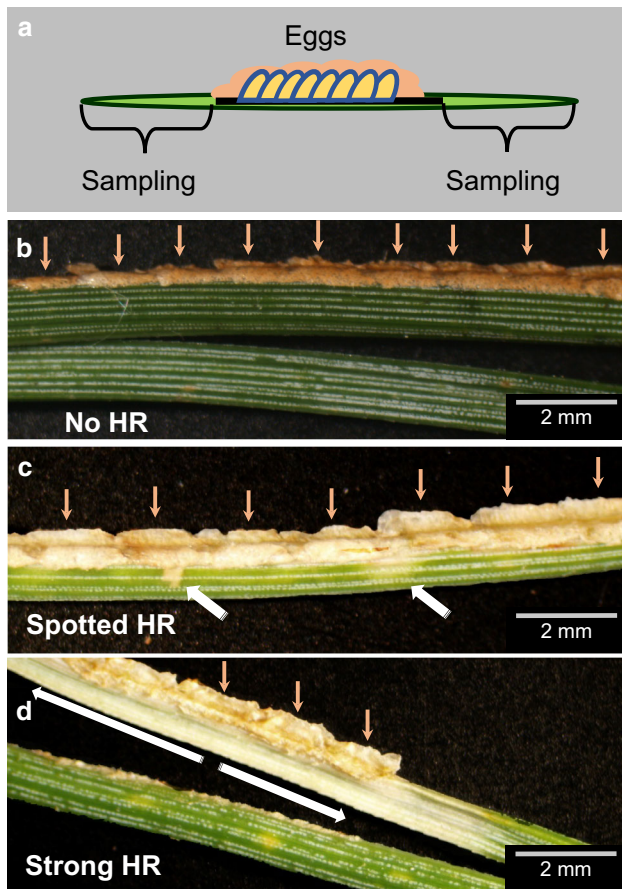


Fig. 1 Needles of *Pinus sylvestris* with *Diprion pini* eggs. **a** Schematic drawing of an egg-deposited needle with yellow eggs laid in a row. Eggs are enclosed in oviduct secretion (blue) and covered on top by secretion of the accessory reproductive glands (ARG) of *D. pini* females (orange). Sampling denotes the site of sampling of tissue from egg-deposited needles for further measurement of enzyme activities. **b–d** Photos of pine needles with *D. pini* eggs covered by brownish ARG secretion. Each single egg in a row is marked by an orange arrow. Whitish needle tissue indicates HR-like pine responses (marked by white arrows)

Materials and methods

Plants and insects

All experiments were conducted with *Pinus sylvestris* L. Plant material was collected from trees growing in forests in the surroundings of Berlin, Germany. We reared the pine sawfly *Diprion pini* L. in our laboratory according to the published protocols for sawfly species (Bomposch and Ramakers 1976; Eichhorn 1976); only the sawfly larvae are herbivorous upon *P. sylvestris*, while the adults do not feed anymore.

For the experiments, we used pine branches of at least 10-year-old trees because younger trees have never been observed to be infested by *D. pini* (Brauns 1991). The branches were kept in the laboratory under conditions as

described in our previous studies on pine responses to sawfly eggs (Mumm and Hilker 2006, and references therein) until used for the experiments.

Determination of frequency of HR-like symptoms

To determine the proportion of egg-deposited needles that show HR-like symptoms, a pine twig was offered to four female and four male *D. pini* individuals for mating and oviposition. The insects were removed from the twig after 24 h, and the number of needles with eggs was counted. In total, we treated 10 twigs. Needles were inspected for development of HR-like symptoms for 12–14 days (= egg incubation time).

Histochemical staining of pine needles for analysis of hydrogen peroxide accumulation and peroxidase activity in response to sawfly eggs

To obtain visible hints on hydrogen peroxide accumulation and peroxidase activity in *P. sylvestris* needles in response to egg deposition by *D. pini*, we used pine needles with 1-, 3- and 12-day-old *D. pini* eggs for staining experiments. We prepared cross sections of these needles by using a razor blade. The freshly hand-cut sections were directly incubated for 30 min in a staining solution composed of 1% 10 mg/ml 3,5,3',5'-tetramethylbenzidine (TMB)–HCl (Sigma) solved in dimethyl sulfoxide (Roth) in a 50 mM Tris–acetate buffer (Roth; pH 5.0); blue staining of tissue is based on the hydrogen peroxide dependent peroxidase-mediated oxidation of TMB (Olson and Varner 1993; Ros-Barceló et al. 2002). For direct staining of hydrogen peroxide, sections were incubated for ~1 h in 4% potato starch (Sigma) in 0.1 M potassium iodide (KI, Roth; pH 5.0) solution (Ros-Barceló et al. 2002) which stains tissue with hydrogen peroxide accumulations brownish. For control, egg-free pine needles were sliced and stained by the same method.

Photos of stained sections were taken by using a photo stereomicroscope (Olympus Research Stereo SZH10; Olympus E-330 camera). Image processing (Figs. 1, 2) by zooming, mirroring and adjusting brightness to the same level was done using the software GIMP 2.8.

Plant treatments

To determine the activities of pine enzymes, a branch was taken from 10 different trees. These 10 branches were provided with tap water and acclimated for 3 days in a climate chamber (20 °C, 18:6 h, L:D, and 100 μmol photons m⁻² s⁻¹) before the treatments. All experimental treatments were started at the beginning of the 10th hour of the long day cycle to account for possible circadian rhythm effects on

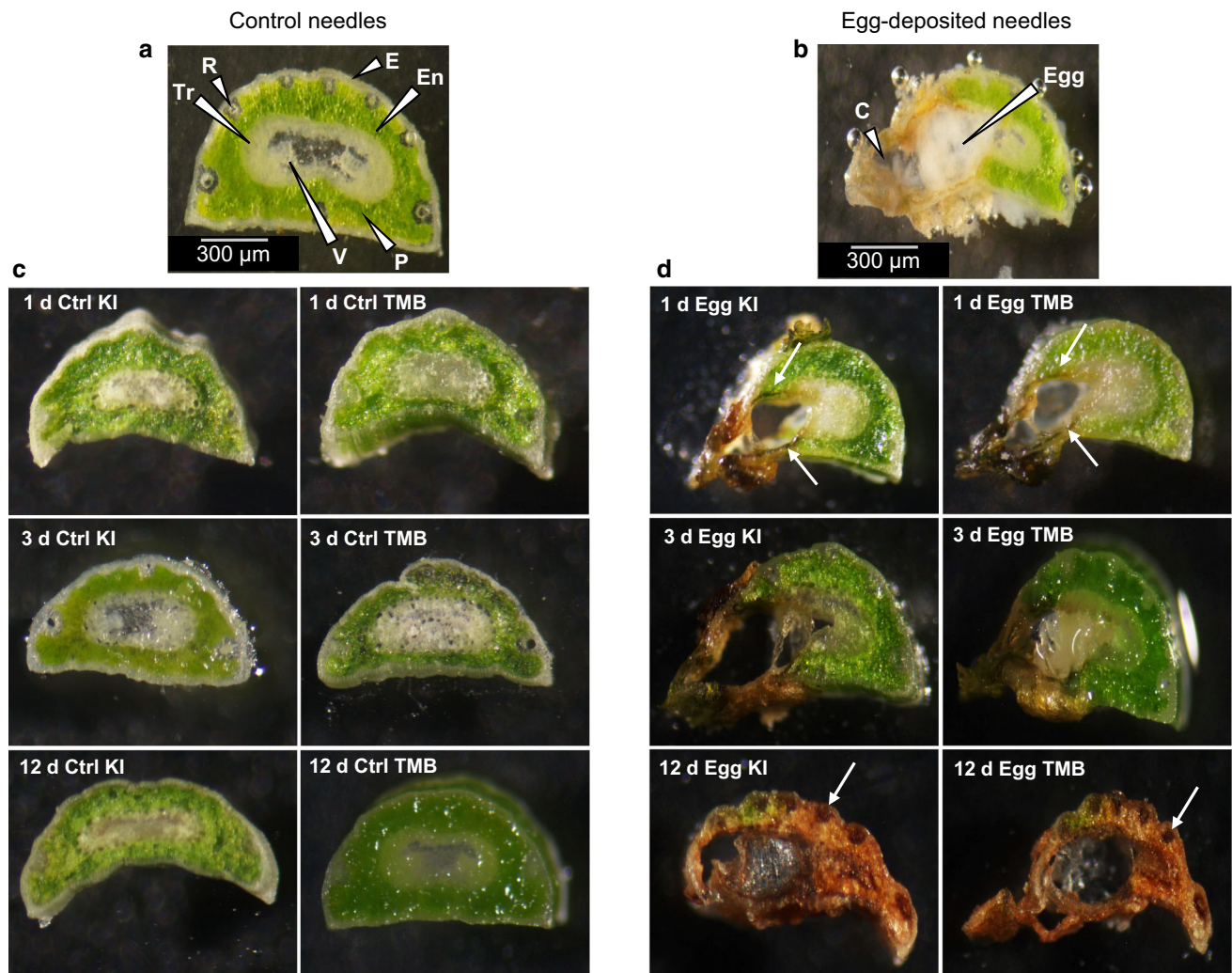


Fig. 2 H_2O_2 detection in cross sections of *Pinus sylvestris* needles. **a** Egg-free unwounded needle without staining. E, epidermis; En, endodermis; P, parenchyma; Tr, transfusion tissue; V, vascular bundle; R, resin canal; **b** Egg-deposited needle without staining. The sawfly *D. pini* female slits a pine needle with the sclerotised ovipositor and inserts eggs deeply into the pine needle tissue after disruption of the epidermis, parenchymatic tissue and endodermis. Eggs are laid in a row into a longitudinally slit pine needle (compare Fig. 1). Eggs inside the needle are enclosed by *D. pini* oviduct secretion which is not distinguishable in the cross section from egg tissue. The opening of the needle at the site of egg insertion is covered

by *D. pini* secretion from the accessory reproductive glands. This secretion is here referred to as “covering” (C) secretion and visible as brownish mass. **c** and **d** Stained cross sections of egg-free control needles (Ctrl) and egg-deposited (egg) needles. KI: starch/potassium iodide for direct H_2O_2 staining. TMB: 3,5,3',5'-tetramethylbenzidine for staining of H_2O_2 linked to peroxidase activity. Photos of egg-deposited needles were taken 1, 3 and 12 days after oviposition, and photos of egg-free needles were taken at equivalent time points. White arrows in **d** point to blue/brown staining of H_2O_2 close to the insect eggs (1 day) and to lignified tissue around resin ducts (12 days)

ROS enzyme activity (Lai et al. 2012). For the treatments, we detached five twigs (about 30 cm long) from one large branch. A twig was used for one of the five treatments described below. Using plant material from one branch for the comparison of treatment effects within a single experimental run was a way to control for constitutive variation of traits which is well known in *P. sylvestris* (e.g. Gershenson and Croteau 1991). In total, we analysed $n = 10$ pine twigs for each enzyme, treatment and time point.

We studied the effect of the following plant treatments on activities of ROS-processing enzymes in pine needles:

1. *Egg deposition* Four female and four male *D. pini* were added to a pine twig for a period of 24 h. After this treatment period, a twig carried at least four egg masses, at maximum 12.

In addition to this treatment of pine needles by natural insect deposition, we conducted several artificial treatments that mimicked different aspects of the insect oviposition:

2. *Wounding* Because a pine sawfly does not only release eggs on a needle, but slits a pine needle longitudinally during oviposition and inserts the eggs in a row into

the slit needle, we were interested in elucidating the effects of only this ovipositional wounding on pine ROS-processing enzymes. Therefore, we slit pine needles with an insect pin in a similar way as a *D. pini* female slits a pine needle while ovipositing (compare Hilker et al. 2002a). We wounded eight pine needles per twig.

3. **Oviduct secretion + wounding** Because a pine sawfly encloses its eggs in oviduct secretion, the pine needle tissue is not in direct contact with the insect egg shell, but with this secretion. Therefore, we studied whether the secretion affects pine ROS-processing enzyme activity and applied 1 µl of a sample of *D. pini* sawfly oviduct secretion dissolved in Ringer solution (Merck; pH 7.2) on each artificially wounded needle [see treatment (2) “wounding”; eight needles per twig]. The oviduct secretion samples were obtained by dissection of *D. pini* females as described earlier (Hilker et al. 2005). In total, each twig received oviduct secretion dissected from four females.
4. **Ringer + wounding** To control for the effect of Ringer solution that has been used for treatment (3) “oviduct secretion” on pine enzyme activity, we wounded eight pine needles per twig as described for treatment (2) and applied 1 µl Ringer into each wound.
5. For control, we also determined enzyme activities of needles from untreated pine twigs.

Sampling pine needle tissue

We harvested the treated needles and respective needles from control twigs in liquid nitrogen for enzyme activity analysis and kept them frozen at -80°C until analysis. While we used entire needles of treatment (2)–(5) for the enzyme assays, we cut out and sampled egg-free tissue of locally egg-laden needles when studying enzyme activity of needles of treatment (1) “egg deposition” (Fig. 1a). We did so for the treatment “egg deposition” to focus on pine tissue enzyme activities, and to exclude enzyme activities that might be also attributable to the eggs.

For determination of pine needle enzyme activities in dependence of the time after treatments, needles of differently treated twigs were collected at following time points: 6 h, 1, 3 and 12 days after treatment ($n = 10$ twigs for each enzyme, treatment and each time point) except for the “egg deposition” treatment. Pine needles from egg-deposited twigs were harvested 1, 3 and 12 days after start of the treatment. We did not sample pine tissue of this treatment after 6 h because pine sawflies needed about a day to produce several egg depositions per twig. The incubation time of *D. pini* eggs lasts about two weeks.

Hence, larvae are close to hatch from eggs at the sampling time point 12 days.

Sampling insect tissue

To determine ROS-scavenging CAT activity in different tissues and secretions of adult *D. pini* females, 1 to 5-day-old females were dissected. All samples were transferred into Ringer solution and frozen at -80°C until measured.

Eggs were dissected from the oviduct to determine the enzymatic activity of eggs without any secretions. One egg sample contained 75 eggs dissected from five females (15 eggs taken from each female). A sample of oviduct secretion which is enveloping the eggs was obtained by dissecting the oviducts of five *D. pini* females. In addition to “wrapping” her eggs with oviduct secretion, a *D. pini* female applies a further secretion on top of the longitudinally slit needle with the eggs inside. We refer to this secretion as “covering secretion” (Fig. 1a) which is produced in the accessory reproductive glands of the females. We dissected this abdominal accessory reproductive gland of an unmated *D. pini* female (= one sample). Haemolymph control samples were collected because all dissected samples were inevitably contaminated with haemolymph. The third pair of legs of a *D. pini* female was cut off at the coxa. Haemolymph that emerged from the experimentally inflicted injuries was sucked into a glass capillary which was attached to a pipette with a small rubber bulb at the end (haemolymph from five females = one sample). As tissue controls, head and thorax were dissected from five *D. pini* females (= one sample).

Enzyme assays: general

All measurements of pine enzyme activities were performed in statistical blocks, i.e. all treatments of one plant of a specific collection time point were measured on the same 96 well plate. Enzyme activities were recorded spectrophotometrically by using the Multiscan[®] GO Microplate Spectrophotometer (Thermo Scientific). Except for measurements of APX activity, all sample extracts that have been prepared as described below were kept frozen at -80°C until measurement; freezing does not affect the activity of enzymes of the antioxidant system (Murias et al. 2005). Sample extracts that were used for the determination of APX activity were measured immediately after extraction (without freezing them) because of the lability of the enzyme (Asada 1992).

NADPH oxidase activity in pine needle tissue

Plant extracts were prepared according to the method of Rojas et al. (2012). Needles were powdered under liquid

nitrogen in a precooled mortar. The needle powder (100 mg) was used for further analysis and extracted in 2 ml extraction buffer consisting of 0.25 M sucrose (Roth), 50 mM Hepes–KOH (Roth; pH 7.2), 3 mM Na₂ EDTA (Roth), 1 mM dithiothreitol (Roth), 0.6% polyvinylpyrrolidone (Sigma), 3.6 mM L-cysteine (Roth), 0.1 mM MgCl₂ (Roth), and SigmaFast™ complete EDTA-free proteinase inhibitor cocktail tablets (Sigma) and filtered through cheesecloth. The filtrate was centrifuged at 4 °C at 10,000×g for 45 min. Supernatants were ultracentrifuged at 203,000×g for 60 min. The resulting pellets were resuspended in 200 µl 10 mM Tris–HCl (Roth; pH 7.4).

NADPH oxidase activity (EC 1.6.3.1) of pine needles was measured by monitoring absorbance in the course of reduction of 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilid–sodium (XTT) (Life Technologies) over a period of 60 min. Absorbance was measured every 20 min at 492 nm starting at time point zero. The reaction mixture consisted of 240 µl of 50 mM Tris–HCl (pH 7.4), 0.5 mM XTT and 100 mM NADPH (Roth) and 10 µl pine extract. A blank sample consisted of 10 µl 10 mM Tris–HCl (pH 7.4). All measurements were performed as technical triplicates. Rates of superoxide production were calculated with an extinction coefficient of $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, blank corrected, and expressed per minute per milligram of pine needle protein.

SOD activity in pine needle tissue

To measure SOD (EC 1.15.1.1) activity, needle extracts were powdered under liquid nitrogen in a precooled mortar. The needle powder (50 mg) was extracted by addition of 500 µl 100 mM potassium phosphate buffer (Roth; pH 7.8), 5% polyvinylpyrrolidone and 0.5% Triton X-100 (Roth). The extract was vortexed and placed on ice for 15 min; this step was repeated once. Thereafter, the extract was centrifuged at 20,817×g, 4 °C for 20 min. The collected supernatant was centrifuged again for 10 min under the same conditions to remove particulate matter. The final supernatant was desalted using Sephadex G-25 columns (GE Healthcare) which were equilibrated with the same buffer as used for extraction, but polyvinylpyrrolidone was omitted. The desalted crude extract was diluted 1:5 with the buffer used for equilibration of Sephadex columns and then used for determination of SOD activity.

The activity measurement was adapted to 96 well plates from a recently published protocol (Popović et al. 2016). A 5 µl aliquot of Sephadex desalted crude pine needle extract was mixed with 245 µl reaction buffer consisting of 50 mM potassium phosphate buffer (pH 7.8), 0.66 mM diethylenetriaminepentaacetic acid (DTPA; Roth), 10 mM L-methionine (Roth), 33 µM nitroblue tetrazolium

(Roth) and 3.3 µM riboflavin (Roth). Plates were kept in the dark until measurement. The reaction was initiated by transferring the plates under fluorescent lamps (100 µm photons m⁻² s⁻¹) for 3 min. Absorbance was measured at 560 nm. To calculate SOD activity in pine needles from absorbance data, an absorbance standard curve was ascertained by pipetting 5 µl of an SOD standard ranging from 1000 to 0 U/ml on each plate. SOD standard was prepared from recombinant bovine SOD (Sigma) in equilibration buffer as described above for the pine needle extract for these measurements. All measurements were performed as technical triplicates. SOD activities of pine needles were calculated by use of the standard curve and expressed as units per gram fresh weight.

APX activity in pine needle tissue

To measure APX (EC 1.11.1.11) activity, needle extracts were prepared as described for the SOD assays, except for addition of 5 mM ascorbic acid (Roth) to the extraction and equilibration buffer.

For measurements we adapted a protocol described by Gillespie et al. (2011) to 96 well plates. 10 µl undiluted, desalted crude pine extract were mixed with 80 µl assay buffer consisting of 50 mM potassium phosphate buffer (pH 7.8), 0.5 mM ascorbic acid and 0.2 mM DTPA. A blank sample consisted of 10 µl equilibration buffer. The measurements began immediately after the addition of 10 µl 20 mM hydrogen peroxide (Roth). Oxidation of ascorbate was monitored at 290 nm for 90 s, measured in 5-s intervals. Due to a high turnover, each row on the 96 well plate was measured sequentially with its own blank to account for pipetting time errors. All measurements were performed as technical triplicates. Ascorbic acid turnover was calculated using a molar extinction coefficient of $2800 \text{ M}^{-1} \text{ cm}^{-1}$, blank corrected and expressed as nmol hydrogen peroxide turnover per minute per milligram of protein.

CAT activity in pine needle tissue

To measure CAT (EC 1.11.1.6) activity, needle extracts were prepared as described above for measuring SOD activity and diluted 1:5 with equilibration buffer.

CAT activity was determined according to the procedure described by Noctor et al. (2016). Desalted crude extract (10 µl) was mixed with 170 µl reaction buffer (100 mM potassium phosphate pH 7.5, 1 mM DTPA). A blank sample consisted of 10 µl equilibration buffer. The reaction was started by addition of 20 µl 0.2 M hydrogen peroxide solution. Decrease in hydrogen peroxide content was monitored for 2 min at 240 nm. All measurements were performed as technical triplicates. CAT activity was

calculated using a molar extinction coefficient of $40 \text{ M}^{-1} \text{ cm}^{-1}$, blank corrected and expressed as μmol hydrogen peroxide degradation per minute per milligram of protein.

CAT activity in insect tissue

Egg extracts were prepared by homogenizing a sample (with 75 eggs) in $50 \mu\text{l}$ Ringer solution using a pestle. The homogenate was centrifuged ($3350\times g$, 10 min, 4°C), and the supernatant of fresh extracts was used for the enzyme assay.

Oviduct extracts were obtained by transferring dissected oviducts of five females to $20 \mu\text{l}$ ice-cold Ringer solution. To remove oviduct cell fragments, samples were centrifuged ($20,814\times g$, 20 min, 4°C), and the supernatant containing the oviduct secretion was used for the analysis. The pellet with oviduct cell fragments was resuspended in $100 \mu\text{l}$ 70 mM potassium phosphate buffer (pH 6.6) containing 0.1% Triton X-100, and the cells were broken by using a syringe. Fresh extracts were used for the assay.

Furthermore, we addressed the question whether oviduct secretion keeps CAT activity after release by the sawfly female. Therefore, we prepared the extracts as described above, but prior to the enzyme assay we kept them stored in open vials for 3 days under the conditions used for pine needle treatment with fresh oviduct extract. We chose a time period of 3 days since previous studies have shown that application of oviduct secretion kept in Ringer solution and stored frozen can elicit a change of pine needle volatiles 3 days after application into slit pine needles; the pine needles treated with this oviduct secretion released volatiles that were attractive to egg parasitoids (Hilker et al. 2005). Here, we were interested in elucidating whether the oviduct secretion also displays ROS processing activity 3 days after release from the females.

To prepare an extract of the accessory reproductive gland of a *D. pini* female, the dissected gland was transferred to $50 \mu\text{l}$ ice-cold Ringer solution and centrifuged as described for oviduct secretion sample. The supernatant was used for analysis.

A haemolymph extract was prepared by adding $20 \mu\text{l}$ ice-cold Ringer solution to the haemolymph collected from five *D. pini* females. The sample was centrifuged as described for the oviduct secretion sample, and the supernatant was analysed.

For control, five heads (thoraxes) of *D. pini* females were homogenised in 300 (500) μl ice-cold Ringer using a pestle. The homogenate was centrifuged ($3350\times g$, 10 min, 4°C), and the supernatant was used for the assays.

We determined insect CAT activity by a different method than the one applied for pine needle CAT activity, and used the procedure described by Johansson and Borg (1988). We did so because the method described by Johansson and Borg does not apply the chelating agent DTPA which was used in the plant CAT assay. As yet we do not know whether

addition of a chelating agent would affect the insect's enzyme activities. The *D. pini* oviduct secretion has so far only been shown to elicit defence responses in pine needles when it was kept in Ringer solution without any further chelating agents (e.g. Hilker et al. 2005).

For the determination of insect CAT activity, each of the insect extracts ($5 \mu\text{l}$) described above was mixed with $50 \mu\text{l}$ methanol (100% v/v) (Roth) and $55 \mu\text{l}$ 250 mM potassium phosphate/sodium hydroxide buffer (Roth; pH 7.0). The reaction was initiated by addition of $10 \mu\text{l}$ 90 mM hydrogen peroxide. The mixture was shaken for 20 min, and the reaction was terminated by the addition of $50 \mu\text{l}$ 7.8 M KOH (Roth). We added $100 \mu\text{l}$ 34.2 mM Purpald[®] (Sigma) in 480 mM HCl (Roth), and the mixture was incubated for 10 min on a shaker. Furthermore, $50 \mu\text{l}$ 65.2 mM potassium periodate (Sigma) was added, and the sample was incubated for 5 min. The absorbance of the purple formaldehyde adduct was measured at 550 nm. All measurements were performed as technical triplicates. CAT activity was calculated using a standard curve with formaldehyde and was expressed as μmol hydrogen peroxide degradation per minute per milligram of protein.

Protein determination of pine and insect samples

To refer the pine and insect enzyme activities mentioned above to the respective total protein contents, we determined protein concentrations of pine needles and insect samples by the method described by Smith et al. (1985) using Pierce[®] BCA Protein Assay Kit (Thermo Scientific) and following the protocol of this kit. Extraction buffers or buffers that were applied to solve membrane pellets were also used for preparation of the corresponding standards.

Protein extracts to which ascorbate oxidase measurements were referred to were diluted tenfold with extraction buffer without ascorbate; thus ascorbate in pine needles was diluted to a final concentration of 0.5 mM which was important because ascorbate interferes with BCA Kit measurements. Extraction buffer containing 0.5 mM ascorbic acid was used for the preparation of standards for these measurements.

Statistics

We used R 3.2.0 (R Development Core Team 2015) for all statistical analyses. The packages MASS, multcomp, nlme and PMCMR from the CRAN server (R Development Core Team 2015) were additionally installed.

Pine needle enzyme activities were statistically evaluated separately for each time point. Normal distribution of data was analysed by the Shapiro–Wilk test.

First, we performed a multiple comparison of pine needle enzyme activities across all treatments (including

control needles). Differences between treatments of normally distributed data were analysed by a linear mixed model with treatment and activity as fixed effects and trees as random effects. The estimation method was set to maximum likelihood. If a treatment had a significant effect on enzyme activities, differences between treatments were detected by the post hoc Tukey test. Non-normally distributed data were evaluated by using a Friedman ANOVA. If significant effects between treatments were detected, they were further evaluated by a post hoc pairwise Wilcoxon–Wilcoxon test with a Benjamini–Hochberg correction for multiple comparisons.

Furthermore, we conducted pairwise comparisons of pine needle enzyme activities in egg-deposited samples and in untreated control needle samples by a Wilcoxon signed-rank test. We conducted this additional pairwise comparison because pine tissue taken from the naturally egg-deposited needles was located next to the eggs (i.e. directly next to the treated site, compare Fig. 1a), whereas pine needles with the artificial treatments were always analysed as whole needles. By taking the needle tissue next to the egg-deposited site we avoided co-analysis of pine and insect egg enzyme activities, but excluded pine tissue located directly at the egg insertion site (treatment site) of the needle.

Sawfly CAT activity data were not normally distributed (Shapiro–Wilk test). Therefore, differences between CAT activities in different tissues were statistically analysed by a Kruskal–Wallis *H* test. The Dunn test with Benjamini–Hochberg correction was used for post hoc comparisons.

Results

Pine needles respond to sawfly egg deposition by locally limited hydrogen peroxide production

HR-like symptoms were observed in about a quarter of *P. sylvestris* needles infested with *D. pini* eggs (Table 1). While pine needles with freshly laid eggs first showed no

specific symptoms (Table 1, Fig. 1b), they formed some yellowish spots (desiccated tissue) along the egg row about 4 days after egg deposition (Fig. 1c). Later at the end of the egg incubation time (12 days after egg deposition) the yellowish, desiccated tissue is visible along the entire egg row (Fig. 1d). Hence, the intensity of these egg-induced pine responses increases during the egg incubation time (Table 1). No larval hatching was observed in egg-deposited needles showing HR-like symptoms.

Cross sections of egg-free and egg-deposited *P. sylvestris* needles were prepared which visualised production of ROS in response to *D. pini* egg deposition (Fig. 2a, b). The secretion covering the eggs on top is brownish even without staining (Fig. 2b); the secretion shows this brownish colour as soon as it is released by the female. The chemistry of this sawfly secretion, which is produced in the insect female accessory reproductive glands, has not been studied yet. Application of TMB or starch/KI solutions at different time points after egg deposition or at respective time points in the controls revealed that egg-free control needles show no blue/brownish (TMB-reaction) or brown (starch/KI reaction) staining that would indicate hydrogen peroxide production (Fig. 2c). However, the needles showed hydrogen peroxide positive staining very locally in pine tissue that is surrounding 1- and 3-day-old sawfly eggs (Figs. 2d, 1 and 3 days). Twelve days after egg deposition hydrogen peroxide positive staining was additionally visible in vascular tissue and in the resin ducts of egg-deposited *P. sylvestris* needles (Fig. 2d, 12 days).

Pine needle activities of SOD, APX and CAT are affected by sawfly oviposition-associated processes

Pine needle NADPH oxidase activity was not significantly affected by either treatment (Table 2). Neither sawfly natural egg deposition nor any of the processes related to the egg deposition (wounding, release of oviduct secretion) did affect the activity of this ROS-generating enzyme.

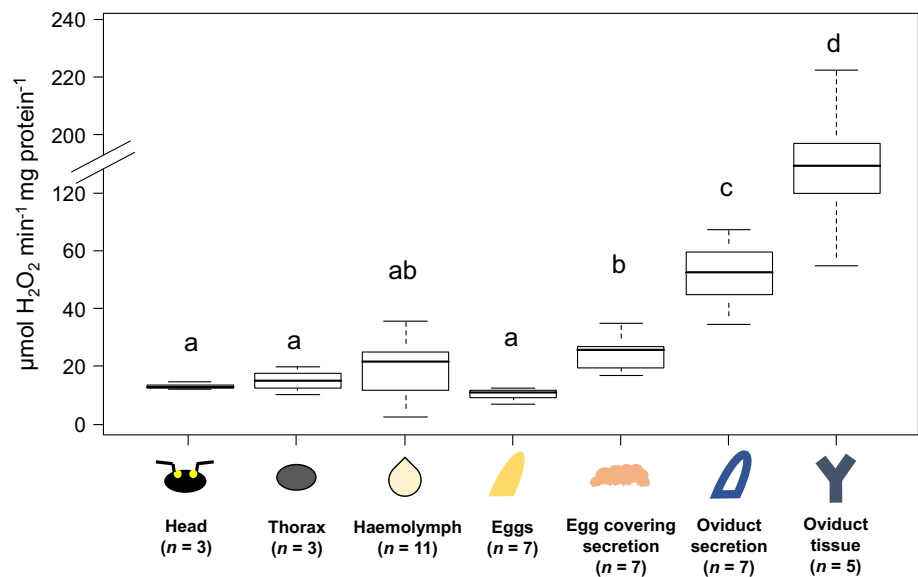
SOD activity in wounded pine needles was significantly lower compared to untreated control needles when

Table 1 Determination of HR-like symptom intensity in *Pinus sylvestris* needles at different time points after egg deposition by *Diprion pini*

Days after egg deposition	% (Mean ± SE) pine needles ^a with		
	No HR-like symptoms	Spotted HR-like symptoms	Strong HR-like symptoms
1	100.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
3	100.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
4	97.7 ± 1.61	2.8 ± 1.60	0.0 ± 0.00
6	89.5 ± 2.71	8.3 ± 2.14	2.3 ± 1.32
12	76.6 ± 4.02	17.8 ± 4.38	5.6 ± 1.40

^a On average each of the 10 trees analysed carried 17.3 (±2.26 SE) needles with eggs

Fig. 3 Catalase activity in freshly sampled *Diprion pini* tissues and secretions (median, interquartile range, minimum/maximum). Statistical differences evaluated by Kruskal–Wallis *H* test. Different letters above boxes indicate significant differences ($P < 0.05$) evaluated by Dunn-test with Benjamini–Hochberg correction



determined 6 h after treatment. Even though wounded pine needles treated with Ringer solution or oviduct secretion also showed slightly lower SOD activities than untreated controls, their activities did not significantly differ from the control. In contrast, 3 and 12 days after treatments SOD activity was significantly higher in wounded needles treated with Ringer and oviduct secretion. The tissue samples from egg-deposited needles showed also slightly higher SOD activity than the controls at day 3 and 12 after treatment, but these activities did not significantly differ from the untreated and artificial controls when performing a multiple comparison including all sample types (Table 2). However, when conducting a pairwise comparison of SOD activities in egg-deposited needles and untreated control needles, we see a significant increase in SOD activity 3 and 12 days after egg deposition.

APX activity was significantly reduced 12 days after all artificial pine needle treatments when compared to control needles. Naturally egg-deposited needles showed a moderate decrease in APX activity as well; however, this did not significantly differ from the APX activities in the other samples, neither when conducting a multiple comparison across all sample types nor in a pairwise comparison with the control (Table 2).

A multiple comparison of CAT activities across all sample types revealed that the activity of this enzyme was significantly reduced in artificially wounded needles and in egg-deposited needles 1 day after treatment when compared to untreated control needles. Three days after treatment, CAT activity was also significantly reduced in wounded needles with oviduct secretion or with Ringer in a multiple comparison. Egg-deposited needles showed a decrease in CAT activity at this time (3 days) only when

compared pairwise with the untreated control needle sample (Table 2).

For all analysed enzymes we observed that especially the activity levels in untreated controls determined after 6 h differed from those measured after 1, 3 and 12 days in control plants. This may be due to diurnal activity changes of ROS-processing enzymes (Lai et al. 2012; Carvalho et al. 2013). All treatments started at the 10th hour of an 18 h-long light cycle in the climate chamber. Thus, samples taken 1, 3 and 12 days after treatments were harvested nearly at ‘noon’ of the day. However, samples taken 6 h after treatment (and at respective time in the controls) were collected almost in the end of the light period. The very low CAT activity detected in control pine needles at the sampling time point 6 h and the higher CAT activities in these needles at time points 1, 3 and 12 days matches results of CAT activity measurements in *A. thaliana*. This plant changes its activity during a day with a peak of activity at noon and lower activities at sunset and sunrise as well as at night (Lai et al. 2012).

Pine sawfly oviduct secretion has strong ROS-scavenging CAT activity

To determine whether *D. pini* females protect their eggs from hydrogen peroxide produced by pine needles in response to egg deposition, we measured CAT activity in the eggs, in the secretion enveloping the eggs (the oviduct secretion), in the tissue producing the secretion (the oviduct), and in the covering secretion applied on top of the eggs on the needle outside as well as other body tissues. Both freshly dissected oviduct tissue and oviduct secretion

Table 2 Activities of ROS-processing enzymes in *Pinus sylvestris* needles after different treatments and time points

Time	Natural Egg Deposition	Artificial Pine Needle Treatments				P value
		Control	Wounding	Ovi.secretion + Wounding	Ringer + Wounding	
NADPH oxidase activity: ROS producing [nmol O ₂ min ⁻¹ mg protein ⁻¹]						
6 h ¹	Not tested -	(3.38 - 5.41) 4.07	(4.64 - 6.41) 5.74	(3.81 - 5.11) 4.25	(4.59 - 5.28) 4.88	0.553
1 d ¹	(4.54 - 6.33) 5.48	(4.09 - 6.63) 5.59	(3.64 - 5.37) 4.50	(4.42 - 6.68) 5.24	(3.80 - 6.67) 5.89	0.982
3 d ¹	(4.83 - 6.28) 5.60	(4.77 - 8.43) 5.58	(2.71 - 4.96) 3.52	(3.03 - 6.43) 5.67	(2.94 - 8.27) 5.10	0.090
12 d ¹	(5.29 - 9.30) 6.68	(7.36 - 9.55) 8.41	(2.41 - 6.71) 3.13	(2.57 - 7.00) 4.55	(1.83 - 5.96) 4.38	0.052
Superoxide dismutase activity: ROS mediating [units g fresh weight ⁻¹]						
6 h ¹	Not tested -	(7.43 - 10.30)^a 10.04	(6.26 - 6.55)^b 6.40	(6.63 - 9.38)^{ab} 6.74	(6.35 - 9.70)^{ab} 8.42	0.015
1 d ²	(5.07 - 6.05) 5.35	(4.98 - 6.07) 5.61	(5.37 - 7.52) 6.29	(4.70 - 7.20) 6.19	(5.69 - 6.92) 5.89	0.188
3 d ¹	*(4.00 - 6.19)^{ab} 5.33	(2.73 - 4.61)^a 3.53	(2.53 - 4.98)^{ab} 3.63	(5.77 - 6.97)^b 6.28	(4.90 - 7.64)^b 5.61	0.006
12 d ²	*(4.57 - 6.68)^{ab} 5.36	(3.68 - 4.70)^a 4.16	(4.48 - 5.89)^a 5.44	(5.80 - 7.99)^b 6.99	(5.71 - 7.10)^b 6.38	0.001
Ascorbate peroxidase activity: ROS degrading [nmol H ₂ O ₂ min ⁻¹ mg protein ⁻¹]						
6 h ²	Not tested -	(560.2 - 809.9) 639.1	(713.9 - 833.8) 767.5	(632.0 - 758.7) 692.1	(652.0 - 855.0) 656.6	0.276
1 d ¹	(509.4 - 551.4) 535.5	(467.1 - 581.7) 515.5	(487.9 - 563.8) 529.1	(501.9 - 574.7) 522.8	(453.7 - 548.3) 538.9	0.620
3 d ¹	(487.3 - 547.4) 526.9	(393.8 - 570.5) 496.3	(476.3 - 527.7) 509.9	(463.9 - 556.1) 500.4	(334.2 - 522.7) 457.2	0.565
12 d ²	(394.8 - 490.8)^{ab} 466.1	(463.0 - 650.8)^a 537.3	(336.5 - 493.7)^b 400.4	(262.2 - 84.6)^b 311.7	(318.2 - 394.0)^b 364.6	0.001
Catalase activity: ROS degrading [μmol H ₂ O ₂ min ⁻¹ mg protein ⁻¹]						
6 h ¹	Not tested -	(14.66 - 47.57) 31.04	(23.51 - 59.93) 47.08	(30.21 - 56.88) 47.47	(25.20 - 51.15) 44.53	0.175
1 d ²	** (16.66 - 55.19)^b 32.55	(41.05 - 84.68)^a 50.89	(19.09 - 47.24)^b 38.60	(33.94 - 66.83)^{ab} 53.74	(30.61 - 54.43)^{ab} 40.58	0.001
3 d ¹	*(35.13 - 54.04)^{ab} 45.04	(48.15 - 74.49)^a 67.03	(34.08 - 53.79)^b 40.69	(29.81 - 62.55)^b 42.31	(31.86 - 55.65)^b 38.47	0.027
12 d ¹	(37.64 - 60.89) 49.75	(51.30 - 77.86) 57.19	(35.89 - 48.93) 43.69	(40.21 - 52.91) 47.50	(40.72 - 54.58) 50.75	0.355

Interquartile ranges/medians are given. $n = 10$. Bold numbers: Significant difference ($P \leq 0.05$) between all natural and artificial treatments (incl. control) evaluated by a Friedman ANOVA for non-normally distributed data (indicated by “1” in column “time”) or a linear mixed model (LMM) for normally distributed data (indicated by “2” in column “time”)

P values given were obtained by Friedman ANOVA or by a LMM (lowest treatment effect value). Different uppercase letters after data: significant differences ($P \leq 0.05$) between treatments evaluated by a post-hoc Tukey test for normally distributed data and a post-hoc Wilcoxon–Wilcox test with Benjamini–Hochberg correction for non-normally distributed data

Asterisks (labelling some “natural egg deposition” data): significant differences ($* P \leq 0.05$; $** P \leq 0.01$) between the treatments “natural egg deposition” and “control” when evaluated by a Wilcoxon signed-rank test. Green (red) marking: enzyme activity significantly decreased (increased) when compared to the “control”. Light green (light red) marking: enzyme activity significantly decreased (increased) in pairwise comparison between the treatments “natural egg deposition” and “control” (Wilcoxon signed-rank test)

showed strong CAT activity, while CAT activity was significantly lower in the covering secretion and in the eggs dissected from the ovaries. The CAT activity in eggs dissected from the ovaries did not differ from CAT activities in other control tissue of *D. pini* females (head, thorax, haemolymph) (Fig. 3).

We also addressed the question whether oviduct secretion keeps its CAT activity after having been released from the female. Therefore, we studied the CAT activity of oviduct secretion 3 days after dissection from females; after dissection, the secretion had been kept under the same abiotic conditions as the treated pine

needles. No CAT activity was detectable in these secretion samples.

Discussion

Plants can defend themselves against insect egg deposition, an initial step of herbivore attack, by the formation of necrotic tissue at the oviposition site, thus causing desiccation or detachment of eggs from the plant (Hilker and Fatouros 2016). No knowledge has been available as yet on the activities of ROS-processing enzymes involved in the formation of these HR-like symptoms. Our study shows that *P. sylvestris* responds to sawfly egg deposition by a change in the activity of ROS-processing enzymes. One and three days after egg deposition, CAT activity was significantly reduced in *P. sylvestris* needles deposited with eggs of the sawfly *D. pini*. Reduction in activity of this hydrogen peroxide degrading enzyme was obviously not specifically triggered by the eggs or egg-associated oviduct secretion, but by the ovipositional wounding that a sawfly inflicts with its ovipositor to a needle while laying the eggs. In contrast, neither natural egg deposition nor ovipositional wounding elicited changes in the activity of the ROS generating NADPH oxidase (Fig. 4).

Wounding of plants has been shown to result in ROS accumulation and increased NADPH oxidase activity (Bi and Felton 1995; Wu and Baldwin 2010; Minibayeva et al. 2015). However, wounding of a plant by insects feeding continuously on the entire leaf or canopy is different from the spatially and temporally restricted

wounding associated with insect egg deposition. Similar to an oviposition-associated wounding, also wounding of plants by gall-inducing insects is locally restricted to a distinct, small site where the gall is formed. Plants can defend against gall-inducing insects by the formation of necrotic tissue at the site where the galling larva starts feeding and by lignification of the gall (Hilker et al. 2002b; Chen 2008); these defensive responses against gall inducers have been suggested to be a result of ROS accumulation (Oliveira et al. 2016). When Liu et al. (2010) studied responses of wheat to attack by a gall midge, they found that ROS accumulation of this plant in response to the gall midge infestation was not due to increased NADPH oxidase activity, but probably to class III peroxidases. A study of responses of the model plant *A. thaliana* to extracts of crushed *Pieris brassicae* eggs revealed that mutants deficient of the NADPH oxidases RBOHD or RBOHF show similar accumulation of ROS as wild-type *A. thaliana* plants, thus indicating NADPH oxidase independent production of ROS in response to this treatment (Gouhier-Darimont et al. 2013). Accumulation of hydrogen peroxide in *P. sylvestris* in response to *D. pini* egg deposition (and associated ovipositional wounding) could neither be attributed to increased NADPH oxidase activity.

A possible explanation for hydrogen peroxide accumulation in egg-deposited *P. sylvestris* needles is that formation of this ROS is increased via an NADPH oxidase independent pathway. Hydrogen peroxide is generated by several enzymes which include—in addition to the key enzyme NADPH oxidase—e.g. peroxisomal glycolate oxidase, xanthine oxidase, urate oxidase, sulphite oxidase, oxalate oxidase and amine oxidase (Lane 2002; Gupta et al. 2016). Amine oxidases are also well known to play a role in the formation of hypersensitive cell death; these enzymes oxidise polyamines, such as putrescine or spermidine, which accumulate in response to phytopathogen infection; hydrogen peroxide is produced by these oxidations (Angelini et al. 2007; Jiménez-Bremont et al. 2014). Furthermore, a study by Roach et al. (2015) indicates that di-amine oxidases also play a significant role in plant responses to wounding.

Another explanation for hydrogen peroxide accumulation in egg-deposited pine needles is that the formation of this ROS does not increase, but that its degradation decreases (Mittler et al. 2004; Torres 2010). This explanation for hydrogen peroxide accumulation in egg-deposited pine needles is supported by the finding that these needles showed reduced CAT activity 1 and 3 days after oviposition. APX activity was significantly reduced in wounded needles 12 days after treatment, while APX activity in the egg-deposited needles decreased by trend at this time point after treatment. Interestingly, the plant

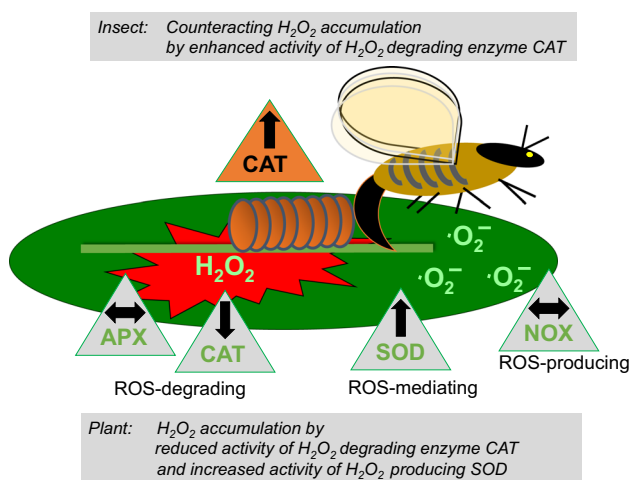


Fig. 4 Proposed model of hydrogen peroxide turnover in *Pinus sylvestris* tissue in response to *Diprion pini* egg deposition/ovipositional wounding. APX, ascorbate peroxidase; CAT, catalase; SOD, superoxide dismutase producing H_2O_2 from the superoxide anion; NOX, NADPH oxidase. The bold green line in the schematic green needle indicates the ovipositional wounding inflicted to pine needle tissue by the sharp sclerotised ovipositor of *D. pini* females

hormone salicylic acid (SA) is known to inhibit APX and CAT in plants (Durner and Klessig 1995, 1996). Accumulation of SA and activation of SA responsive defence genes has not been determined in egg-deposited pine needles so far, but was observed in *A. thaliana* locally at the site of egg deposition by *P. brassicae* (Gouhier-Darimont et al. 2013).

Changes in SOD activities in response to the treatments varied dependent on the treatment and time point of measurement. Induction of SOD is a general phenomenon related to stress in plants (Alscher et al. 2002). Thus, the significant increase of SOD activities in wounded needles treated with oviduct secretion or Ringer solution (3 and 12 days after treatment) were expected because of the wound stress. Additionally, when comparing egg-deposited needles directly to untreated control needles we can also observe a significant increase in SOD activity, indicating an accumulation of hydrogen peroxide at the site of the egg deposition. However, the lack of changes in SOD activities in artificially wounded samples and the decrease in SOD activity shortly after wounding (6 h) remains elusive.

Our study suggests that the differential regulation of ROS-mediating enzyme activities in response to egg deposition was very localised. When we performed a multiple comparison of enzyme activities across all sample types, several significant changes that were found in response to wounding of *P. sylvestris* needles were detectable only by trend in response to *D. pini* egg deposition. This may be due to the type of tissue sampling. While we measured enzyme activities in the entire wounded needles, we sampled only the tissue right next to the eggs in the egg-deposited needles to avoid measuring egg-inherent enzyme activities. However, when we conducted a pairwise comparison of enzyme activities in egg-deposited and untreated control needles, we can see similar significant effects of natural egg deposition and the artificial treatments, except for APX. Very local responses to egg deposition were also found in *A. thaliana* deposited with eggs of the butterfly *P. brassicae*; differential regulation of genes were most pronounced in leaf tissue directly below the eggs (Little et al. 2007). Staining of pine needle sections also showed a very localised accumulation of hydrogen peroxide at the sawfly egg—pine needle interface (Fig. 2d).

Hydrogen peroxide accumulation in egg-deposited needles may on the one hand serve defence signalling that is initiating stress-related transcription of defence gene networks (Mittler et al. 2011). On the other hand, plant ROS accumulation in response to the eggs might directly harm the developing eggs. Furthermore, increased ROS levels kill plant cells and thus, might reduce humidity of the needle tissue in which *D. pini* eggs are embedded. This consequence of ROS accumulation may lead to desiccation of *D. pini* eggs. Indeed, our study

showed that those needles which form HR-like symptoms can kill all the eggs laid into them; no larvae hatched from eggs in these needles. If pine tissue does not form clearly visible HR-like symptoms and eggs survive, egg-induced changes in ROS-mediating enzyme activities may also affect the hatching larvae which start feeding at the natal site. Staining of the egg-deposited needles revealed the presence of hydrogen peroxide and peroxidase activity in the vascular tissue and resin ducts of needle sections (Fig. 2d). This is a strong indication of ongoing lignification processes which are mediated by hydrogen peroxide and peroxidase activity in xylem tissue (Barceló 1998; Ros-Barceló et al. 2002). Lignified and desiccated needle tissue may also negatively affect neonate *D. pini* larvae which suffer increased mortality when starting their larval development at the site where they hatch as compared to larvae which start feeding on egg-free needles (Beyaert et al. 2012). Hence, oviposition-induced formation of pine hydrogen peroxide or of other ROS may be involved in harming *D. pini* eggs in various ways.

Defence of *P. sylvestris* against insect eggs by ROS accumulation is not left unanswered by *D. pini*. Oviduct secretion of *D. pini* enclosing the eggs and oviduct tissue producing the secretion showed very high ROS-scavenging activity, while CAT activity in eggs dissected from *D. pini* ovaries was not higher than in non-reproductive *D. pini* tissue. In another hymenopteran species, the parasitic wasp *Cotesia vestalis*, eggs showed increased peroxidase and CAT activity after egg deposition into host larvae (Hao et al. 2012). However, it remained unclear whether these enzyme activities in *Cotesia* eggs are induced in response to host immune activities or whether these are due to maternal secretions that *Cotesia* wasps attach to their eggs when ovipositing (e.g. Glatz et al. 2004). Other studies showed that CAT plays a central role in protecting oocytes and the early embryo from ROS damage (DeJong et al. 2007; Diaz-Albiter et al. 2011; Sim and Denlinger 2011). Our results suggest that the sawfly *D. pini* relies on protecting the offspring from plant ROS by external egg secretions rather than by egg-inherent ROS-scavenging enzyme activity. However, enhanced CAT activity was only found in freshly released oviduct secretion, whereas oviduct secretion kept at the same experimental conditions as the treated pine needles had lost its strong CAT activity 3 days after dissection (release) from *D. pini* females. The high CAT activity of freshly released oviduct secretion may avert the negative effects of a ROS burst as initial response of pine to the ovipositional wounding by *D. pini* females (Fig. 4).

In conclusion, our study shows close ROS-related interactions between *P. sylvestris* and *D. pini* egg deposition. This plant and insect species share a long common evolutionary time since the Paleogene when the sawfly genus *Diprion* evolved (O'Reilly et al. 2015). Pine species need to have evolved survival mechanisms which limit

sawfly populations because high population densities of the gregariously feeding *D. pini* larvae may severely damage entire pine forests (e.g. Lyytikäinen-Saarenmaa et al. 2002). A defensive response of pine against the initial step of attack by *D. pini*, the egg deposition, is accumulation of ROS. Future studies need to address whether in addition to the detected decreased activity of CAT also ROS-generating enzymes other than NADPH oxidase are involved in ROS accumulation in egg-deposited pine needles. The pine response to egg deposition is counteracted by high CAT activity in *D. pini* oviduct secretion that is associated with the eggs and ovipositional wounding. The sawfly eggs and associated secretions may contain in addition to CAT further ROS-scavenging enzymes (Felton and Summers 1995; Mathews et al. 1997) which contribute to limiting the negative effects of ROS produced by pine needles.

Author contribution statement NB designed the experiments, performed all plant enzyme measurements and related data analysis. UTK performed all insect tissue enzyme measurements and related data analysis. NB and UTK took the photos. MH conceptualized and supervised the study, participated in its design and coordination. NB drafted the manuscript, and MH and UTK assisted in drafting and revising the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

All animals in this study were treated according to legal guidelines of the European Union and the Federal Republic of Germany as well as according to institutional guidelines of Freie Universität Berlin.

Conflict of interest The authors declare they have no conflict of interest and consented in submitting and publishing this study.

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References

- Almagro L, Gómez Ros LV, Belchi-Navarro S et al (2009) Class III peroxidases in plant defence reactions. *J Exp Bot* 60:377–390. doi:10.1093/jxb/ern277
- Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Bot* 53(1331–134):1. doi:10.1093/jexbot/53.372.1331
- Angelini R, Tisi A, Rea G et al (2007) Involvement of polyamine oxidase in wound healing. *Plant Physiol* 146:162–177. doi:10.1104/pp.107.108902
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399. doi:10.1146/annurev.arplant.55.031903.141701
- Asada K (1992) Ascorbate peroxidase—a hydrogen peroxide-scavenging enzyme in plants. *Physiol Plant* 85:235–241. doi:10.1111/j.1399-3054.1992.tb04728.x
- Barceló AR (1998) Hydrogen peroxide production is a general property of the lignifying xylem. *Ann Bot* 82:97–103. doi:10.1006/anbo.1998.0655
- Barros J, Serk H, Granlund I, Pesquet E (2015) The cell biology of lignification in higher plants. *Ann Bot* 115:1053–1074. doi:10.1093/aob/mcv046
- Baxter A, Mittler R, Suzuki N (2014) ROS as key players in plant stress signalling. *J Exp Bot* 65:1229–1240. doi:10.1093/jxb/ert375
- Beyaert I, Köpke D, Stiller J et al (2012) Can insect egg deposition “warn” a plant of future feeding damage by herbivorous larvae? *Proc R Soc B* 279:101–108. doi:10.1098/rspb.2011.0468
- Bi JL, Felton GW (1995) Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J Chem Ecol* 21:1511–1530. doi:10.1007/BF02035149
- Bombosch S, Ramakers PMJ (1976) Zur Dauerzucht von *Gilpinia hercyniae* Htg. *Z Pflanzenk Pflanzen* 83:40–44
- Bozhkov PV, Lam E (2011) Green death: revealing programmed cell death in plants. *Cell Death Differ* 18:1239–1240. doi:10.1038/cdd.2011.86
- Brauns A (1991) Taschenbuch der Waldinsekten, 4th edn. Gustav Fischer Verlag, Stuttgart
- Caverzan A, Passaia G, Rosa SB et al (2012) Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. *Genet Mol Biol* 35:1011–1019. doi:10.1590/S1415-47572012000600016
- Chen MS (2008) Inducible direct plant defense against insect herbivores: a review. *Insect Sci* 15:101–114. doi:10.1111/j.1744-7917.2008.00190.x
- Coll NS, Epple P, Dangl JL (2011) Programmed cell death in the plant immune system. *Cell Death Differ* 18:1247–1256. doi:10.1038/cdd.2011.37
- da Carvalho KS, Pinheiro HA, Festucci-Buselli RA et al (2013) Diurnal changes in leaflet gas exchange, water status and antioxidant responses in *Carapa guianensis* plants under water-deficit conditions. *Acta Physiol Plant* 35:13–21. doi:10.1007/s11738-012-1043-6
- DeJong RJ, Miller LM, Molina-Cruz A et al (2007) Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA* 104:2121–2126. doi:10.1073/pnas.0608407104
- Diaz-Albiter H, Mitford R, Genta FA et al (2011) Reactive oxygen species scavenging by catalase is important for female *Lutzomyia longipalpis* fecundity and mortality. *PLoS One* 6:e17486. doi:10.1371/journal.pone.0017486
- Durner J, Klessig DF (1995) Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. *Proc Natl Acad Sci USA* 92:11312–11316. doi:10.1073/pnas.92.24.11312
- Durner J, Klessig DF (1996) Salicylic acid is a modulator of tobacco and mammalian catalases. *J Biol Chem* 271:28492–28501. doi:10.1074/jbc.271.45.28492
- Eichhorn O (1976) Dauerzucht von *Diprion pini* L. (Hym.: Diprionidae) im Laboratorium unter Berücksichtigung der Fotoperiode. *Anz Schädlingskd Pfl* 49:38–41. doi:10.1007/BF02342165
- Fatouros NE, Lucas-Barbosa D, Weldegergis BT et al (2012) Plant volatiles induced by herbivore egg deposition affect insects of

- different trophic levels. *PLoS One* 7:e43607. doi:10.1371/journal.pone.0043607
- Fatouros NE, Paniagua Voirol LR, Drizou F et al (2015) Role of large cabbage white butterfly male-derived compounds in elicitation of direct and indirect egg-killing defenses in the black mustard. *Front Plant Sci* 6:1–9. doi:10.3389/fpls.2015.00794
- Felton GW, Summers CB (1995) Antioxidant systems in insects. *Arch Insect Biochem Physiol* 29:187–197. doi:10.1002/arch.940290208
- Gershenzon J, Croteau R (1991) Terpenoids. In: Rosenthal GA, Berenbaum MR (eds) *Herbivores: their interactions with secondary plant metabolites*, 2nd edn. Academic Press, San Diego, pp 165–219
- Gillespie KM, Rogers A, Ainsworth EA (2011) Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (*Glycine max*). *J Exp Bot* 62:2667–2678. doi:10.1093/jxb/erq435
- Glatz R, Schmidt O, Asgari S (2004) Isolation and characterization of a *Cotesia rubecula* bracovirus gene expressed in the lepidopteran *Pieris rapae*. *J Gen Virol* 85:2873–2882. doi:10.1099/vir.0.80307-0
- Gouhier-Darimont C, Schmiesing A, Bonnet C et al (2013) Signalling of *Arabidopsis thaliana* response to *Pieris brassicae* eggs shares similarities with PAMP-triggered immunity. *J Exp Bot* 64:665–674. doi:10.1093/jxb/ers362
- Gupta K, Sengupta A, Chakraborty M, Gupta B (2016) Hydrogen peroxide and polyamines act as double edged swords in plant abiotic stress responses. *Front Plant Sci* 7:1–19. doi:10.3389/fpls.2016.01343
- Hao Z-P, Zhao J-R, Yuan Z-Q et al (2012) Influence of photoperiod on hydrogen peroxide metabolism during diapause induction in *Cotesia vestalis* (Haliidae) (Hymenoptera: Braconidae). *J Kansas Entomol Soc* 85:206–218. doi:10.2317/JKES120318.1
- Hilker M, Fatouros NE (2015) Plant responses to insect egg deposition. *Annu Rev Entomol* 60:493–515. doi:10.1146/annurev-ento-010814-020620
- Hilker M, Fatouros NE (2016) Resisting the onset of herbivore attack: plants perceive and respond to insect eggs. *Curr Opin Plant Biol* 32:9–16. doi:10.1016/j.pbi.2016.05.003
- Hilker M, Kobs C, Varama M, Schrank K (2002a) Insect egg deposition induces *Pinus sylvestris* to attract egg parasitoids. *J Exp Biol* 205:455–461
- Hilker M, Rohfritsch O, Meiners T (2002b) The plant's response towards insect egg deposition. In: Hilker M, Meiners T (eds) *Chemecology of insect eggs and egg deposition*. Blackwell Publishing Ltd, Oxford, pp 205–233
- Hilker M, Stein C, Schröder R et al (2005) Insect egg deposition induces defence responses in *Pinus sylvestris*: characterisation of the elicitor. *J Exp Biol* 208:1849–1854. doi:10.1242/jeb.01578
- Jiménez-Bremont JF, Marina M, de la Guerrero-González ML et al (2014) Physiological and molecular implications of plant polyamine metabolism during biotic interactions. *Front Plant Sci* 5:95. doi:10.3389/fpls.2014.00095
- Johansson LH, Borg LA (1988) A spectrophotometric method for determination of catalase activity in small tissue samples. *Anal Biochem* 174:331–336
- Köpke D, Schröder R, Fischer HM et al (2008) Does egg deposition by herbivorous pine sawflies affect transcription of sesquiterpene synthases in pine? *Planta* 228:427–438. doi:10.1007/s00425-008-0747-8
- Köpke D, Beyaert I, Gershenzon J et al (2010) Species-specific responses of pine sesquiterpene synthases to sawfly oviposition. *Phytochemistry* 71:909–917. doi:10.1016/j.phytochem.2010.03.017
- Lai AG, Doherty CJ, Mueller-Roeber B et al (2012) CIRCADIAN CLOCK-ASSOCIATED 1 regulates ROS homeostasis and oxidative stress responses. *Proc Natl Acad Sci USA* 109:17129–17134. doi:10.1073/pnas.1209148109
- Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* 48:251–275. doi:10.1146/annurev.arplant.48.1.251
- Lane BG (2002) Oxalate, germins, and higher-plant pathogens. *IUBMB Life* 53:67–75. doi:10.1080/10399710290038954
- Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by pierid butterflies triggers defense responses in *Arabidopsis*. *Plant Physiol* 143:784–800. doi:10.1104/pp.106.090837
- Liu X, Williams CE, Nemacheck JA et al (2010) Reactive oxygen species are involved in plant defense against a gall midge. *Plant Physiol* 152:985–999. doi:10.1104/pp.109.150656
- Lyytikäinen-Saarenmaa P, Lyytikäinen-Saarenmaa P, Tomppo E (2002) Impact of sawfly defoliation on growth of Scots pine *Pinus sylvestris* (Pinaceae) and associated economic losses. *Bull Entomol Res* 92:137–140. doi:10.1079/BER2002154
- Mathews MC, Summers CB, Felton GW (1997) Ascorbate peroxidase: a novel antioxidant enzyme in insects. *Arch Insect Biochem Physiol* 34:57–68. doi:10.1002/(SICI)1520-6327(1997)34:1<57::AID-ARCH5>3.0.CO;2-T
- Meng X, Zhang S (2013) MAPK cascades in plant disease resistance signaling. *Annu Rev Phytopathol* 51:245–266. doi:10.1146/annurev-phyto-082712-102314
- Mimibayeva F, Beckett RP, Kranner I (2015) Roles of apoplastic peroxidases in plant response to wounding. *Phytochemistry* 112:122–129. doi:10.1016/j.phytochem.2014.06.008
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7:405–410. doi:10.1016/B978-044482650-3/50031-6
- Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. *Trends Plant Sci* 9:490–498. doi:10.1016/j.tplants.2004.08.009
- Mittler R, Vanderauwera S, Suzuki N et al (2011) ROS signaling: the new wave? *Trends Plant Sci* 16:300–309. doi:10.1016/j.tplants.2011.03.007
- Morel JB, Dangel JL (1997) The hypersensitive response and the induction of cell death in plants. *Cell Death Differ* 4:671–683. doi:10.1038/sj.cdd.4400309
- Mumm R, Hilker M (2006) Direct and indirect chemical defence of pine against folivorous insects. *Trends Plant Sci* 11:351–358. doi:10.1016/j.tplants.2006.05.007
- Mumm R, Schrank K, Wegener R et al (2003) Chemical analysis of volatiles emitted by *Pinus sylvestris* after induction by insect oviposition. *J Chem Ecol* 29:1235–1252. doi:10.1023/A:1023841909199
- Mur LA, Kenton P, Lloyd AJ et al (2008) The hypersensitive response; the centenary is upon us but how much do we know? *J Exp Bot* 59:501–520. doi:10.1093/jxb/erm239
- Murias M, Rachtan M, Jodynis-Liebert J (2005) Effect of multiple freeze-thaw cycles of cytoplasm samples on the activity of antioxidant enzymes. *J Pharmacol Toxicol Methods* 52:302–305. doi:10.1016/j.vascn.2005.03.002
- Navrot N, Rouhier N, Gelhaye E, Jacquot JP (2007) Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiol Plant* 129:185–195. doi:10.1111/j.1399-3054.2006.00777.x
- Noctor G, Mhamdi A, Foyer CH (2016) Oxidative stress and antioxidative systems: recipes for successful data collection and interpretation. *Plant, Cell Environ* 39:1140–1160. doi:10.1111/pce.12726
- O'Brien JA, Daudi A, Butt VS, Bolwell GP (2012) Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta* 236:765–779. doi:10.1007/s00425-012-1696-9

- O'Reilly JE, dos Reis M, Donoghue PCJ (2015) Dating tips for divergence-time estimation. *Trends Genet* 31:637–650. doi:[10.1016/j.tig.2015.08.001](https://doi.org/10.1016/j.tig.2015.08.001)
- Oliveira DC, Isaias RMS, Fernandes GW et al (2016) Manipulation of host plant cells and tissues by gall-inducing insects and adaptive strategies used by different feeding guilds. *J Insect Physiol* 84:103–113. doi:[10.1016/j.jinsphys.2015.11.012](https://doi.org/10.1016/j.jinsphys.2015.11.012)
- Olson PD, Varner JE (1993) Hydrogen peroxide and lignification. *Plant J* 4:887–892. doi:[10.1046/j.1365-313X.1993.04050887.x](https://doi.org/10.1046/j.1365-313X.1993.04050887.x)
- Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a swiss army knife. *Plant Cell Rep* 24:255–265. doi:[10.1007/s00299-005-0972-6](https://doi.org/10.1007/s00299-005-0972-6)
- Popović M, Šuštar V, Gričar J et al (2016) Identification of environmental stress biomarkers in seedlings of European beech (*Fagus sylvatica*) and Scots pine (*Pinus sylvestris*). *Can J For Res* 46:58–66. doi:[10.1139/cjfr-2015-0274](https://doi.org/10.1139/cjfr-2015-0274)
- R Development Core Team (2015) R: A language and environment for statistical computing
- Reape TJ, Molony EM, McCabe PF (2008) Programmed cell death in plants: distinguishing between different modes. *J Exp Bot* 59:435–444. doi:[10.1093/jxb/erm258](https://doi.org/10.1093/jxb/erm258)
- Reczek CR, Chandel NS (2015) ROS-dependent signal transduction. *Curr Opin Cell Biol* 33:8–13. doi:[10.1016/j.ceb.2014.09.010](https://doi.org/10.1016/j.ceb.2014.09.010)
- Reymond P (2013) Perception, signaling and molecular basis of oviposition-mediated plant responses. *Planta* 238:247–258. doi:[10.1007/s00425-013-1908-y](https://doi.org/10.1007/s00425-013-1908-y)
- Roach T, Colville L, Beckett RP et al (2015) A proposed interplay between peroxidase, amine oxidase and lipoxygenase in the wounding-induced oxidative burst in *Pisum sativum* seedlings. *Phytochemistry* 112:130–138. doi:[10.1016/j.phytochem.2014.06.003](https://doi.org/10.1016/j.phytochem.2014.06.003)
- Rojas CM, Senthil-Kumar M, Wang K et al (2012) Glycolate oxidase modulates reactive oxygen species-mediated signal transduction during nonhost resistance in *Nicotiana benthamiana* and *Arabidopsis*. *Plant Cell* 24:336–352. doi:[10.1105/tpc.111.093245](https://doi.org/10.1105/tpc.111.093245)
- Ros-Barceló A, Pomar F, López-Serrano M et al (2002) Developmental regulation of the H₂O₂-producing system and of a basic peroxidase isoenzyme in the *Zinnia elegans* lignifying xylem. *Plant Physiol Biochem* 40:325–332. doi:[10.1016/S0981-9428\(02\)01376-1](https://doi.org/10.1016/S0981-9428(02)01376-1)
- Sim C, Denlinger DL (2011) Catalase and superoxide dismutase-2 enhance survival and protect ovaries during overwintering diapause in the mosquito *Culex pipiens*. *J Insect Physiol* 57:628–634. doi:[10.1016/j.jinsphys.2011.01.012](https://doi.org/10.1016/j.jinsphys.2011.01.012)
- Smith PK, Krohn RI, Hermanson GT et al (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76–85. doi:[10.1016/0003-2697\(85\)90442-7](https://doi.org/10.1016/0003-2697(85)90442-7)
- Torres MA (2010) ROS in biotic interactions. *Physiol Plant* 138:414–429. doi:[10.1111/j.1399-3054.2009.01326.x](https://doi.org/10.1111/j.1399-3054.2009.01326.x)
- Wu J, Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. *Annu Rev Genet* 44:1–24. doi:[10.1146/annurev-genet-102209-163500](https://doi.org/10.1146/annurev-genet-102209-163500)