# Evaluation of the Oxidative Burst in Suspension Cell Culture of *Phaseolus vulgaris*

Kerstin Janisch and Harald Schempp*

Technical University of Munich, WZW Center of Life Sciences, Institute of Phytopathology, Am Hochanger 2, 85350 Freising, Germany. Fax: +49(0)81 61 71 45 38.
E-mail: h.schempp@lrz.tum.de

* Author for correspondence and reprint requests

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Plants respond to the attack of pathogens with the oxidative burst, a production of reactive oxygen species (ROS). In this work a cell culture suspension of *Phaseolus vulgaris* was used to investigate the oxidative burst triggered by a conidia suspension of different races of *Colletotrichum lindemuthianum.* As a defence response of the cells a two-phase peak was observed with all used races of *Colletotrichum lindemuthianum*, varying only in the produced amounts of hydrogen peroxide. Findings with additives such as superoxide dismutase (SOD), diphenyleneiodonium (DPI) and catalase gave rise to the conclusion that more superoxide radicals were produced than be detectable with Amplex® Red as hydrogen peroxide. It is assumed that the conversion of the superoxide radical is spontaneous and not driven via a cell-derived superoxide dismutase. The addition of low-molecular cell wall components (ergosterol, glucosamine, galactosamine) showed clearly that compounds like this act as elicitors and thus are involved in triggering the burst. Furthermore, an evaluation of the metabolizing capacities of hydrogen peroxide of the suspension culture cells revealed the enormous capacity of the cells to detoxify this ROS.

*Key words:* Hydrogen Peroxide Detection, Oxidative Burst, *Phaseolus vulgaris* Suspension Cell Culture

## Introduction

Plants developed several strategies to defend themselves against a wide variety of pathogens. Besides structural resistance and preformed defence substances, there are also induced defence mechanisms, which begin with the recognition of the pathogen. Elicitors, derived either from the cell wall of the pathogen (= exogenous elicitor) and/or from the plant (= endogenous elicitor) bind to receptors of the plant membrane and activate G-proteins and phospholipases. This triggers an influx of $Ca^{2+}$ and $H^+$-ions in the cytoplasm; in cell cultures this influx of protons is measurable as an alkaline shift of the media. $K^+$ and $Cl^-$-ions efflux is observed at the same time (Blumwald *et al.*, 1998; Elstner *et al.*, 1996; Lamb and Dixon, 1997; Mehdy *et al.*, 1996; Wojtaszek, 1997a, b). Followed by these processes is the generation of superoxide radicals and hydrogen peroxide, the 'oxidative burst'. Two possibilities for the generation of the superoxide radicals are discussed:

a) Generation via a membrane-bound NADPH-oxidase, which is activated by phosphorylation: NADPH-oxidase is known from leukocytes and its existence in plants was shown with inhibitors of the animal NADPH-oxidase, such as diphenyleneiodonium (DPI). Conformity between the animal and plant enzyme was also proven with serological tests, gene analyses and amino acid sequencing (Baker and Orlandi, 1995; Blumwald *et al.*, 1998; Elstner *et al.*, 1996; Lamb and Dixon, 1997; Low and Merida, 1996; Mehdy *et al.*, 1996; Murphy and Auh, 1996; Wojtaszek, 1997a, b).

b) Generation via NADH-dependent peroxidases, which are found in the cell wall: Their existence was proven by KCN-inhibition of the oxidative burst (Auh and Murphy, 1995; Baker and Orlandi, 1995; Lamb and Dixon, 1997; Mehdy, 1994; Vera-Estrella *et al.*, 1994a, b; Wojtaszek, 1997b; Wojtaszek *et al.*, 1997).

According to Low and Merida (1996), reactive oxygen species (ROS) are generated in two phases. Phase I, with a duration up to 60 min, is induced by compatible host-pathogen interactions

*Abbreviations:* DPI, diphenyleneiodonium; d.w., dry weight; HR-POD, horseradish peroxidase; ROS, reactive oxygen species; SAR, systemic acquired resistance; SOD, superoxide dismutase.

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shortly after elicitation, whereas phase II only sets on by incompatible host-bacteria interactions about 2 h after elicitation with increased and longer-lasting ROS production (Mehdy, 1994; Wojtaszek *et al.*, 1997). Fungal and chemical elicitors just cause an 'oxidative burst' of phase I shortly (2–15 min) after elicitation (Anderson *et al.*, 1991; Mehdy, 1994; Wojtaszek *et al.*, 1997). The generation of superoxide radicals is depending on the plant species. Bolwell *et al.* (1998) reported that suspension cell cultures of *Rose damascena* generate superoxide radicals via NADPH-oxidase, whereas in bean (*Phaseolus vulgaris*) suspension cell cultures peroxidases are relevant. According to Bestwick *et al.* (1999) both mechanisms exist and operate in plants, but each species exerts its 'preference'. However, superoxide radicals are the starting points for all further developing ROS (hydrogen peroxide, hydroxyl radicals), but they are not necessarily detectable or relevant as a signal to develop resistance by the following processes:

- activation of defence genes by $H_2O_2$ leading to the induction of phytoalexines and PR-proteins,
- stabilisation of the cell wall by oxidative cross linking via hydroxyl proline-rich proteins, tyrosine residues in extensines and ferulic acid in arabinoglucans during the hypersensitive reaction,
- induction of systemic acquired resistance (SAR) by $H_2O_2$ as a discussed messenger compound or by salicylic acid, a likewise necessary component of the SAR,
- killing of the pathogen due to the anti-microbial properties of $H_2O_2$

(Apostol *et al.*, 1989a; Baker and Orlandi, 1995; Elstner *et al.*, 1996; Guo *et al.*, 1997; Lamb and Dixon, 1997; Low and Merida, 1996; Mehdy *et al.*, 1996; Mehdy, 1994; Wojtaszek, 1997a, b; Wojtaszek *et al.*, 1997).

The generated $H_2O_2$ during the oxidative burst is usually detected with luminol or fluorescence spectrometry (dye = Pyranine or Oxonol VI) (Anderson *et al.*, 1991; Apostol *et al.*, 1989a, b; Bolwell *et al.*, 1998; Guo *et al.*, 1997). In this work, the dye 'Amplex® Red' was used to determine the hydrogen peroxide, as it is, according to Zhou *et al.* (1997), more sensitive than other indicators or dyes. A further positive aspect of this assay is the low auto-fluorescence of biological compounds due to the excitation/emission wavelengths of 575/585 nm. In the presence of horseradish peroxidase (HR-POD) the colourless, non-fluorescent dye is oxidized by hydrogen peroxide to the red, fluorescent resorufin. The stoichiometric conversion of Amplex® Red to resorufin by hydrogen peroxide (Fig. 1) is 1:1 at pH 7.4 (Molecular Probes, 1998; Murphy and Auh, 1996). Thus, an increase in the fluorescence of Amplex® Red indicates increasing hydrogen peroxide concentrations in the sample.

In this study conidia suspensions of *Colletotrichum lindemuthianum* were used as elicitors following some work groups which used cell wall preparations of this pathogen (Anderson *et al.*, 1991; Bolwell *et al.*, 1998; Stoibiecki *et al.*, 1996). *C. lindemuthianum* belongs to the phylum of the Deuteromycota, class Coelomycetes and the pathogen causes anthracnose on bean plants (*Phaseolus vulgaris*). The fungus includes 6 races of different pathogenicity, the resistance towards the single races is specific for the cultivar (Hoffmann *et al.*, 1994; Müller and Loeffler, 1992; personal communication: Zinkernagel, 1999). In the case of the used cell culture suspension of the common French bean (*Phaseolus vulgaris*), the pathogenicity/resistance towards the 6 different fungal races is as follows: races $\alpha$ and $\beta$, resistance; race $\lambda$, moderate resistance; races $\delta$ and $\varkappa$, susceptibility.

## Results

The oxidative burst response of the cells induced by five different races of the fungus was studied using fluorescence determination of $H_2O_2$ by conversion of Amplex® Red into the highly fluorescent resorufin (Fig. 1). To characterize the detected ROS, the influence of superoxide dismutase (SOD), DPI and catalase on the measured fluorescence signal was investigated. In this case the oxidative burst was induced by *C. lindemuthianum* race alpha. Furthermore, fungal cell wall components (ergosterol, glucosamine and galactosamine) were used to induce the oxidative burst of the suspension cells. In none of the experiments cell death of the bean cells was observed.

First of all, the bean cells were treated with conidia suspensions of five different races of *C. lindemuthianum* as seen in Fig. 2. All five races induced two-phase oxidative bursts merely varying in the generated $H_2O_2$-amounts. The three *C. lindemuthianum*-races alpha, beta and lambda induced about 5.5 $\mu$mol/g dry weight (d.w.) hydrogen peroxide production within the first peak of the cell burst, whereas elicitation with the races

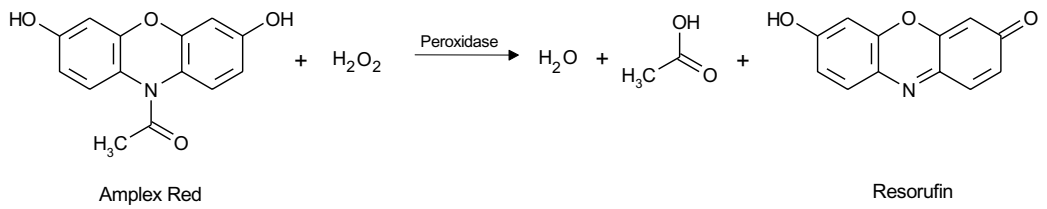


Fig. 1. Conversion of Amplex® Red to resorufin by $H_2O_2$/peroxidase.

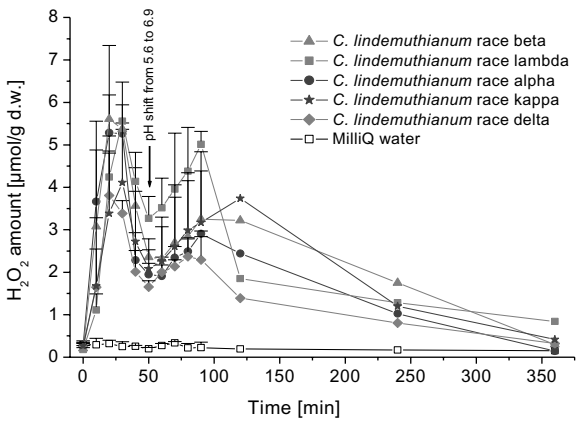


Fig. 2. Bean cells treated with different races of *C. lindemuthianum* ($1 \times 10^4$ conidia/ml).

kappa and delta resulted in a lesser $H_2O_2$ production (about 4.25 $\mu$mol/g d.w., and 4.00 $\mu$mol/g d.w., respectively). The onset of the second peak started 50 min after elicitation for all *C. lindemuthianum*-races used (Fig. 2). The height of the peak was reached 90 min after elicitation, when the races alpha and lambda were used ($H_2O_2$-amounts: 3.0 $\mu$mol/g d.w., and 5.0 $\mu$m o l/g d.w., respectively). Elicitation with race delta resulted in the earliest second peak (maximum reached at 80 min) with the lowest $H_2O_2$-amounts measured (*ca.* 2.5 $\mu$mol/g d.w.). It turned out, that French bean cells treated either with race beta or kappa showed the latest second peak maxima at 100 min and 120 min, respectively, with nearly the same amounts of $H_2O_2$ (3.25 $\mu$mol/g d.w., and 3.75 $\mu$mol/g d.w., respectively). The pH shift shown in Fig. 2 was determined for the elicitation with *C. lindemuthianum* race alpha and indicates an alkalisation of the media during the course of the burst.

When catalase was present in the culture media of elicited cells, the measurable $H_2O_2$-amount decreased to values of about 0.5 $\mu$mol $H_2O_2$/g d.w. (Fig. 3). In the presence of SOD the oxidative burst peaks showed the same course, but measurable $H_2O_2$-amounts increased, reaching about 6.5 $\mu$mol $H_2O_2$/g d.w. for the first peak and nearly 5.0 $\mu$mol $H_2O_2$/g d.w. within the second peak (Fig. 3). In contrast, DPI suppressed the burst showing only a small first peak (maximum $H_2O_2$-amount about 2.0 $\mu$mol/g d.w.) but no second peak. Interestingly, the first peak corresponded to the burst response of the cells when treated with 0.6% ethanol alone as a control (see Fig. 4). Ethanol was used as solvent for DPI and ergosterol, therefore the effect of ethanol in the concentration range used was also tested as a solvent control.

Ergosterol as elicitor caused an early peak in analogy to the first peak of the *C. lindemuthianum* treatment (Fig. 4) with similar $H_2O_2$-amounts (*ca.* 5.25 $\mu$mol/g d.w.). In contrast, glucosamine and galactosamine induced courses of the burst similar to the second peak of the *C. lindemuthianum* elicitation, but with less detectable $H_2O_2$ (maximum

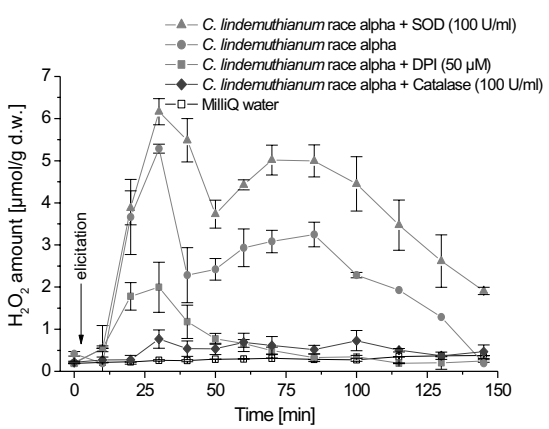


Fig. 3. Bean cells treated with *C. lindemuthianum* ($1 \times 10^4$ conidia/ml) and 50 $\mu$M diphenyleneiodonium (DPI), 100 U/ml superoxide dismutase (SOD), 100 U/ml catalase.

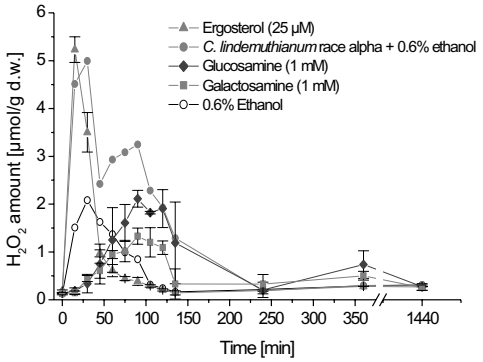


Fig. 4. Bean cells treated with either *C. lindemuthianum* ($1 \times 10^4$ conidia/ml) or 25 $\mu$M ergosterol or 1 mM glucosamine or 1 mM galactosamine.

values: *ca.* 2.25 $\mu$mol/g d.w. for glucosamine and *ca.* 1.5 $\mu$mol/g d.w. for galactosamine; Fig. 4).

To investigate the properties of the bean cells to metabolize $H_2O_2$, defined concentrations of $H_2O_2$ were added to the cells as internal standards and the retrieval of the $H_2O_2$ was assessed after 10 min (Table I), which corresponds to the reading points of all other experiments. In Table I the capacity of the bean cells to metabolize added $H_2O_2$-amounts is shown in comparison to the properties of two tobacco suspension cell cultures (Bel B and Bel W3). In the range from 1 to 25 $\mu$M added $H_2O_2$, the tobacco cell lines show a higher capacity to remove $H_2O_2$ standards. Moreover, in the range of 50 to 250 $\mu$M $H_2O_2$, Bel W3 is still better than the bean cell line, whereas $H_2O_2$ metabolizing activity of Bel B was lower than that of the bean cells (Table I). However, the bean cell line is definitely better in metabolizing high concentrations of $H_2O_2$ (250 $\mu$M–10 m M) than the two tobacco cell lines (Table I).

## Discussion

The elicitation of cell suspension of French bean (*Phaseolus vulgaris*) produces a two-phase oxidative burst. To suggest the second peak to be phase II of an incompatible host-parasite relationship is contrary to Low and Merida (1996) as phase II sets on after about 2 h with an increased and longer enduring $H_2O_2$ production. Considering the pathogenicity/resistance interaction between the used bean suspension culture (common French bean, *Phaseolus vulgaris*) and the single races of *C. lindemuthianum* (races $\alpha$ and $\beta$: resistance; race $\lambda$: moderate resistance; races $\delta$ and $\varkappa$: susceptibility), only the races $\alpha$, $\beta$ and $\lambda$ should produce a phase II. The results of this work are consistent with others (Lamb and Dixon, 1997; Mehdy, 1994; Wojtaszek, 1997b) postulating generally a one-phase peak by fungal pathogens. In accordance to Anderson *et al.* (1991) and Bolwell *et al.* (1998), the oxidative burst of French bean elicited by *C. lindemuthianum* shows the same profile. The observed course of the burst must be due to the used elicitor material. Compared to earlier investigations using less defined cell wall preparations, in this study living conidia suspension was applied. It is assumed that the first peak of the detected oxidative burst is initiated by cell and/or cell wall constituents of the fungus, which solve during the

| Added $H_2O_2$ concentration | Recovered $H_2O_2$ concentration [µM] | | | | | |
|---|---|---|---|---|---|---|
| | Bean cells | | Tobacco cells Bel B | | Tobacco cells Bel W3 | |
| [$\mu$M] | Mean | SD | Mean | SD | Mean | SD |
| 0 | 0.403 | 0.002 | 0.234 | 0.011 | 0.243 | 0.022 |
| 1 | 0.854 | 0.327 | 0.431 | 0.198 | 0.155 | 0.009 |
| 2.5 | 1.021 | 0.163 | 0.347 | 0.060 | 0.173 | 0.048 |
| 5 | 3.505 | 1.246 | 0.357 | 0.015 | 0.213 | 0.030 |
| 10 | 3.213 | 1.097 | 0.332 | 0.021 | 0.213 | 0.030 |
| 25 | 8.909 | 1.579 | 0.472 | 0.025 | 0.177 | 0.094 |
| 50 | 9.978 | 4.335 | 5.683 | 0.454 | 0.381 | 0.164 |
| 100 | 33.50 | 16.34 | 5.767 | 0.437 | 0.435 | 0.115 |
| 250 | 25.96 | 8.292 | 36.67 | 4.252 | 9.384 | 4.777 |
| 500 | 24.17 | 10.47 | 61.50 | 0.866 | 37.17 | 4.041 |
| 1000 | 25.54 | 8.997 | 44.50 | 3.041 | 67.33 | 21.079 |
| 5000 | 188.3 | 48.76 | – | – | – | – |
| 10000 | 176.7 | 54.58 | – | – | – | – |

Table I. Recovery of internal standards of $H_2O_2$ in bean, tobacco Bel B and Bel W3 cells; sample collection at 10 min for bean cells and 15 min for tobacco cells.

preparation of the conidia suspension. The second peak of the burst is therefore the result of the attachment of the conidia to the cells and/or the onset of the metabolism of the conidia releasing elicitors giving an additional stimulus for the cells. The measured pH-shift (Fig. 2) was determined clearly after 60 min, but the alkalisation of the media started already after 25 min of elicitation. Considering a spontaneous pH-dependent dismutation of the superoxide radicals into $H_2O_2$ and $O_2$, the pH increase of about 1 value (5.6–6.9) of the cell media explains the decrease of detected amounts of $H_2O_2$. This assumption is supported by SOD addition (100 units/ml), indicating the spontaneous dismutation of the superoxide radical is the limiting factor of the detectable $H_2O_2$ production (Fig. 3). The production of superoxide radicals occurs over a longer time course (peak II, Fig. 3). The decrease of the measurable $H_2O_2$ in peak II is due to the alkalisation of the media (Fig. 2) and the onward generated superoxide radicals has to be cleared otherwise.

The specificity of Amplex® Red reagent to react with $H_2O_2$ in the presence of horseradish peroxidase as well as that $H_2O_2$ is the detected ROS is proven by the addition of catalase (Fig. 3), which maintains no measurable signal.

According to Wojtaszek (1997a, b) and Bolwell *et al.* (1998) peroxidases are responsible for the oxidative burst in bean cell culture. DPI, an inhibitor of NADPH-oxidase (Benhamou, 1996; Lamb and Dixon, 1997; Wojtaszek, 1997a, b), also inhibits the oxidase function of peroxidases in the used concentration of 50 $\mu$M according to Frahry and Schopfer (1998). Although there is still a small first peak in the presence of DPI (Fig. 3), which is induced by the solvent ethanol (Fig. 4). Ethanol effects the cell membranes and therefore evokes stress signals within the cells leading to a production of $H_2O_2$.

The exact composition of added elicitors is seldom known. This gave rise to the thought that low-molecular fungal cell wall components such as ergosterol, glucosamine or galactosamine might function as elicitors. The addition of ergosterol results in a quick response which is similar to the first peak of the burst after elicitation with conidia (Fig. 4). The compounds glucosamine and galactosamine induce a $H_2O_2$ production after a lag period of about 25 min corresponding to the second peak of the *C. lindemuthianum* burst (Fig. 4). Therefore it is concluded that these fungal cell wall components play a role in the recognition of the pathogen by the cultured suspension cells.

The cells generate $H_2O_2$ already in quiescent state (Table I) as investigations on the metabolizing capacities showed. With about 0.4 $\mu$M $H_2O_2$, the bean cells produce twofold more than the tobacco cells. The tobacco cell line Bel W3 metabolizes the added $H_2O_2$ standards to recovery rates in the nanomolar range up to an added concentration of 100 $\mu$M; for Bel B this accounts up to the concentration of 25 $\mu$M (Table I). The enormous capacity to metabolize added $H_2O_2$ standards can be seen with the bean cells which detoxify the added 5 and 10 mM to about 4% and 2% of the input concentrations (measured: 188 $\mu$M and 177 $\mu$M, respectively; Table I). The metabolizing capacities of the cells do not give any linearity, but there might be a threshold concentration due to an exhausted detoxifying capacity of the suspension cells.

## Materials and Methods

### *Material*

Amplex® Red (10-acetyl-3,7-dihydroxy phenoxazine) was purchased from Molecular Probes. D-(+)-Galactosamine hydrochloride, D-(+)-glucosamine hydrochloride, diphenyleneiodonium (DPI), ergosterol and superoxide dismutase (SOD) were obtained from Sigma, Germany. Hydrogen peroxide was purchased from Merck, Germany. Dimethyl sulfoxide was from Riedel-de Haën, Germany. Catalase and horseradish peroxidase (HRPOD) were obtained from Boeringer Mannheim GmbH, Germany. The colour-fixed indicator sticks pH-Fix 5.1–7.2 were from Macherey-Nagel, Germany.

The fungus *Colletotrichum lindemuthianum* was cultivated at the Institute. The suspension solution of conidia was obtained prior to use by washing the mycelia with MilliQ water. The concentration of the conidia was counted with an haemocytometer and diluted to an appropriate concentration to give a final concentration of $1 \times 10^4$ conidia/ml in the flasks.

The cell culture suspension of *Phaseolus vulgaris* was a kind gift of GSF Neuherberg, Germany.

### *Instruments*

Fluorescence spectrometer: Hitachi F-4500 with Siemens Nixdorf PCD-4H Computer.

Microscope: Leitz Wetzlar SM-Lux.

Haemocytometer: Fisher Scientific Comp. Brightline Hemacytometer.

Table rotary shaker: Kötterman 4010 Schüttler.

*Method*

The results shown are means of four individual experiments (n = 4) unless stated otherwise, standard deviations are given as $\sigma_{n-1}$. The calculations were performed using Microsoft Excel and Microcal Origin 5.0.

Cells were grown in darkness on a rotary shaker at 120 rpm in an iron-free modified 1-B5-media of Gamborg and Phillips (1995) and for all experiments 2-day-old suspension cultures were used as these provided the best response concerning elicitor treatment (data not shown). Growth and media consumption curves were assessed to calculate the dilution factor for the treatments and $H_2O_2$-amount per gram dry weight (d.w.) of the results (data not shown).

The generated $H_2O_2$ was measured using an Amplex® Red assay (for 1 ml, final concentrations): 0.1 M phosphate buffer, pH 7.4, 1 U/ml HRPOD, 100 $\mu$M Amplex® Red, 400 $\mu$l aliquot of cell media without cells. The Amplex® Red dye was first dissolved in DMSO to give a stock solution of 20 mM and then diluted with MilliQ water (bidest. < 0.05 $\mu$S) to the appropriate concentration.

The fluorescence spectrometer settings were: excitation 575 nm; emission 580 nm; slits 5 nm/1 nm; voltage 700 V; time 2 s.

A $H_2O_2$-standard curve with Amplex® Red is linear in the range of 0 to 5.5 $\mu$M $H_2O_2$ (data not shown). The following equation gives the $H_2O_2$-amount per gram dry weight (d.w.), considering the data of the growth curve and dilution factor:

$$H_2O_2\ (\mu\text{mol/g d.w.}) = \{[(F/18.414\ \text{mAU}/\mu\text{mol}) \times d] \times V\}/m$$

where $F$ is the fluorescence (mAU), $d$ is the dilution factor, $V$ is the volume of the media in the flask (l) and $m$ is the dry weight of cells (g).

The sampling was done every 10 min for the first 1.5 to 2 h and 4, 6 and 24 h after elicitor treatment. The time point t = 0 corresponds to the addition of the elicitor if not indicated otherwise. The cells were kept in darkness at 120 rpm on a shaker all the time.

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