Early Detection of *Phoma lingam* Infection in Oilseed Winter Rape Before Visible Symptoms Appear

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A chlorophyll fluorescence technique enabled early detection of disturbances in the activity of the photosynthetic apparatus under *Phoma lingam* infection. The photosynthetic apparatus of leaves and cotyledons exhibited a negative response to *P. lingam* inoculation. The effect disappeared more rapidly in leaves, which were not inoculated directly, than in cotyledons, which were inoculated directly. Photosynthetic apparatus disturbances were detected in cotyledons and leaves as early as 24 h after inoculation. Photosynthetic apparatus activity can be affected by the level of hydrogen peroxide, which in cotyledons probably was an element of the hypersensitive response and in leaves could induce an increase in pathogenesis-related proteins, chitinase and β-1,3-glucanase.

Key words: Blackleg, *Brassica napus*, chlorophyll fluorescence, chitinase, hydrogen peroxide, photosynthetic apparatus, β-1,3-glucanase.

INTRODUCTION

A number of methods are available for assessing the activity of the photosynthetic apparatus under environmental stresses (Lichtenthaler and Miehé, 1997; Zou et al., 2009; Skórska, 2011). Modern fluorometers simultaneously measure several chlorophyll fluorescence parameters characteristic of a particular process of photosystem II (PSII) (Maxwell and Johnson, 2000). Such a complex analysis enables estimation of the quantum photochemical efficiency of PSII, the efficiency of excitation energy utilization in the photosynthesis process, and the level of reaction center opening. Fluorometers also permit analysis of the amount of energy scattered by the photosynthetic apparatus as heat that can signal damage to the photosynthetic apparatus.

From those measurements it is possible to determine whether the photosynthetic apparatus was damaged and at which stage the photochemical processes was disturbed (Bolhar-Nordenkampf and Öquist, 1993; Baker and Rosenqvist, 2004).

Chlorophyll fluorescence imaging is increasingly often used to study the response of the photosynthetic apparatus to biotic environmental factors (Esfeld et al., 1995; Ning et al., 1995; Bowyer et al., 1998). The main advantage of this method is its noninvasiveness. It enables the whole surface of the leaf to be studied, facilitating precise analysis of photosynthetic apparatus functioning in different parts of a leaf surface subjected to a stress (Lichtenthaler and Miehé, 1997). In the case of a biotic stress this may yield additional information on the functioning of the photosynthetic apparatus at the point of inoculation with a fungal pathogen as well as in the immediate vicinity of the inoculated tissue before the first symptoms of the disease become visible (Lai et al., 2011).

Blackleg, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. (vegetative stage: *Phoma lingam* Tode ex Fr. Desm.), is one of the most damaging diseases of oilseed winter rape (Jędryczka, 2007). In our previous study we demonstrated differences in photosynthetic apparatus activity in winter rape seedlings grown on agar medium containing toxic culture of *Phoma lingam* filtrates at 4, 7 and 11 days after inoculation (Hura et al., 2014). Little is known about early disturbances (between days 1 and 4 post-inoculation) of photosynthetic apparatus activity in winter rape seedlings under *Phoma lingam* infection. We suspected that the functioning of the photosynthetic apparatus is disturbed in earlier phases of infection and posited

that it might be correlated with, for example, the hydrogen peroxide level. A hypersensitive response (HR), in this case increased accumulation of reactive oxygen species (ROS) at the infection site, may limit the growth of a fungal pathogen, but ROS can also be toxic to cell structures, including the photosynthetic apparatus (Małolepsza and Urbanek, 2002). Hydrogen peroxide, the most stable ROS, also can freely pass through biological membranes and induce a systemic response, for example an increase of pathogenesis-related (PR) proteins in noninoculated leaves of a plant (Scherer et al., 2005; Bienert et al., 2006).

This study was aimed at early detection of photosynthetic apparatus disturbances before any visible symptoms occur in cotyledons (directly inoculated) and leaves (indirectly inoculated). We also analyzed photosynthetic apparatus activity in leaves with regard to accumulation of hydrogen peroxide and PR proteins. The systemic response was analyzed based on the activity of two types of PR proteins, chitinase and β-1,3-glucanase, participating in defense mechanisms involving destruction of the fungal pathogen's cell wall.

MATERIAL AND METHODS

PLANT GROWTH CONDITIONS

The study used seedlings of oilseed winter rape (*Brassica napus* L.) cv. Lisek, mildly resistant to blackleg disease. The plants were cultivated in plastic pots (5 seedlings per pot, diameter 15 cm, height 18 cm). Each pot was filled with a homogenous mixture of soil, peat and sand (2:1:1 v/v/v). The plants were grown in a greenhouse at $16\pm2°C$, ~60% RH and 80% soil water content under a 12 h photoperiod (PPFD ~180 μmol photons $m^{-2} \cdot s^{-1}$ at leaf surface).

FUNGUS INOCULATION

Sixteen-day-old seedlings (with 2 cotyledons and 2 leaves) were inoculated with *Phoma lingam* spores as described by Jędryczka et al. (1991). The cotyledons were centrally injected (syringe with a needle) with 10 μl inoculum (1.5 × 10 spores·ml) and incubated in shade in a growth chamber at 18°C under 80% RH. Control cotyledons were inoculated with an equal volume of sterile distilled water. The isolate (Ph L5) (Kachlicki and Jędryczka, 1994) from the fungus *Phoma lingam* was obtained from the Institute of Plant Genetics, Polish Academy of Sciences in Poznań.

CHLOROPHYLL FLUORESCENCE

Chlorophyll fluorescence of leaves and cotyledons was measured with a chlorophyll fluorescence imaging system (Fluorcam 700MF, PSI, Brno, Czech Republic), as

described by Nedbal et al. (2000a). Fluorcam v. 3.5 software was used to control the imaging system and process the images. To estimate maximum photochemical efficiency (F_v/F_m), the leaves were adapted to darkness for 20 min, then illuminated, and the photochemical quenching coefficient (q_P), the efficiency of excitation transfer to open PSII centers (F'_v/F'_m), PSII quantum efficiency (Φ_{PSII}), the nonphotochemical quenching coefficient (q_N) and the chlorophyll fluorescence decrease ratio (R_{Fd}) were calculated as described by Wingler et al. (2004). For each growing seedling the data were averaged for the whole leaf and cotyledon separately. The measurements were performed 24, 48, 72, and 96 h after inoculation in 7 replicates (i.e., 1 leaf/1 cotyledon from each of 7 seedlings for each post-inoculation period). Chlorophyll fluorescence was measured from cotyledons directly inoculated with *Phoma lingam* spores, and from indirectly inoculated leaves. The measurements were made at the seedling stage of two cotyledons and two leaves.

DETECTION OF H_2O_2 ACCUMULATION

Hydrogen peroxide was detected as described by Thordal-Christensen et al. (1997). Plant samples were infiltrated with DAB (3,3'-diaminobenzidine) solution for 15 min in darkness under 0.8 MPa pressure. Chlorophyll was removed from leaves and cotyledons by rinsing them several times with hot (60°C) ethanol (96%). The brown spots of H_2O_2 accumulation that appeared on the leaves and cotyledons were photographed and the digital images were saved.

B-1,3-GLUCANASE (EC 3.2.1.39) AND CHITINASE (EC 3.2.1.14)

Activity of β-1,3-glucanase was estimated according to Fink et al. (1988) using Somogyi reagent (Somogyi, 1952) and Nelson reagent (Nelson, 1944). The amount of glucose in the plant extract was determined spectrometrically at 540 nm. β-1,3-glucanase activity is given in katals per gram fresh weight (1 katal corresponds to the formation of 1 mol glucose per second).

Chitinase activity was analyzed according to Legrand et al. (1987). The amount of N-acetyl-glucosamine (Glc-Nac) released from chitin by the enzyme in the plant extract was analyzed spectrometrically at 585 nm. Chitinase activity is expressed in nkatals (nanokatals) per gram fresh weight. The activity of β-1,3-glucanase and chitinase was determined in five replicates.

STATISTICAL ANALYSIS

The significance of differences was determined by Student's t-test at p = 0.05, using Statistica for Windows v. 10.0.

RESULTS

MAXIMUM PHOTOCHEMICAL EFFICIENCY AND THE EFFICIENCY OF EXCITATION TRANSFER TO OPEN PSII CENTERS

After inoculation with *Phoma lingam* spores, between 24 and 96 h the maximum photochemical efficiency of PSII (F_v/F_m) measured in dark-adapted leaves and cotyledons of plants was significantly lower than in control plants (Fig. 1). The F_v/F_m ratio decreased gradually between 24 and 48 h in both leaves and cotyledons, and then increased beginning at 72 h. The increase was more marked in the (indirectly inoculated) leaves.

Inoculation of cotyledon tissues was accompanied by a decrease in the F_v'/F_m' ratio, the parameter indicating the efficiency of excitation transfer to open PSII centers (Fig. 1). Following infection, F_v'/F_m' was lowest at 24 h in leaves and between 24 and 72 h in cotyledons. F_v'/F_m' values began to recover to the control level between 48 and 96 h in leaves, while in cotyledons that rise was observed at 96 h.

PSII QUANTUM EFFICIENCY, PHOTOCHEMICAL QUENCHING COEFFICIENT AND NONPHOTOCHEMICAL QUENCHING

A decrease of PSII quantum efficiency (Φ_{PSII}) in leaves were observed between 24 and 48 h after infection (Fig. 2). Thereafter the Φ_{PSII} values approximated those of control plants. In cotyledons the decline of Φ_{PSII} was much greater; it rose at 96 h, but not to the level observed in control plants.

The pattern of changes under pathogenesis was similar for the photochemical quenching coefficient (q_P): q_P was lower for cotyledons than for leaves (Fig. 2). In leaves it increased considerably between 24 and 48 h, and in cotyledons between 24 and 72 h (Fig. 2). More efficient utilization of excitation energy in leaves, which had not been inoculated directly, was confirmed by decreased q_N at 72 h.

VITALITY INDEX OF THE PHOTOSYNTHETIC APPARATUS

We also found significant differences in the vitality index (R_{fd}) of the photosynthetic apparatus, reflecting the efficiency of the light and dark phases of photosynthesis (Fig. 3). After infection, R_{fd} was lower for up to 48 h in leaves and for up to 72 h in cotyledons, and was lower in cotyledons than in leaves.

H_2O_2 DETECTION

As the control leaves and cotyledons showed similar H_2O_2 levels, only a few representative images are shown (Fig. 4). At 24 h after inoculation with
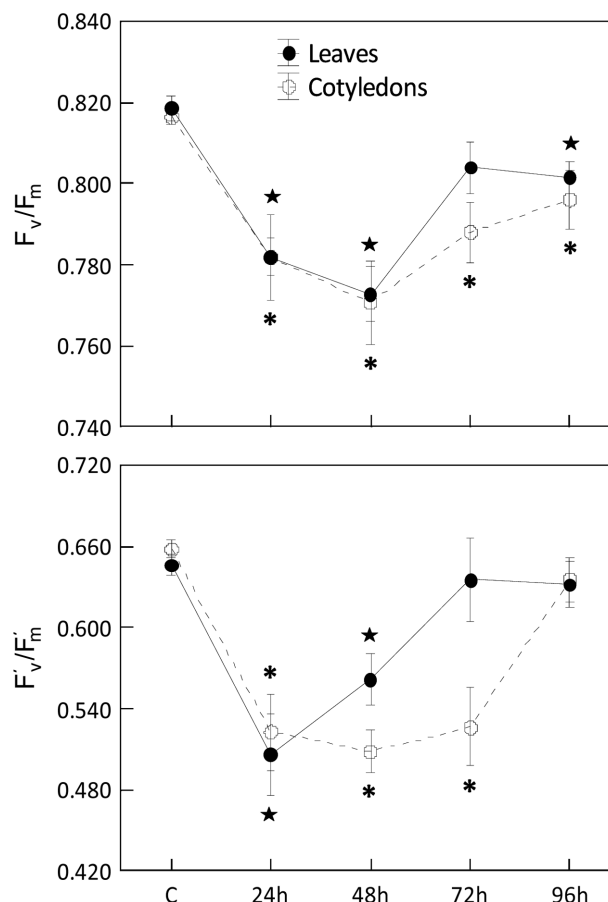

Fig. 1. Changes in F_v/F_m (maximum photochemical efficiency) and F_v'/F_m' (efficiency of excitation transfer to open PSII centers) in leaves and cotyledons observed 24, 48, 72 and 96 h after inoculation. Points represent means ± standard error. C – means for total measurements performed in parallel 24, 48, 72 and 96 h after water inoculation of control plants. For cotyledons *P < 0.05 vs. control, Student's t-test. For leaves ★P < 0.05 vs. control, Student's t-test.

P. lingam spores the hydrogen peroxide concentration in cotyledons was high (brown spots), and higher than in cotyledons inoculated with water. Hydrogen peroxide accumulation in infected cotyledons was also elevated at 48, 72 and 96 h. For indirectly infected leaves there was a marked rise in hydrogen peroxide at 24 h and a smaller one at 48 h. At 72 and 96 h the H_2O_2 levels in infected seedlings were similar to control values.

PATHOGENESIS-RELATED PROTEINS

In leaves the activity of β-1,3-glucanase significantly increased at 48, 72 and 96 h after infection; chitinase activity increased at 72 and 96 h (Fig. 5). The activity of both enzymes in the leaves of seedlings
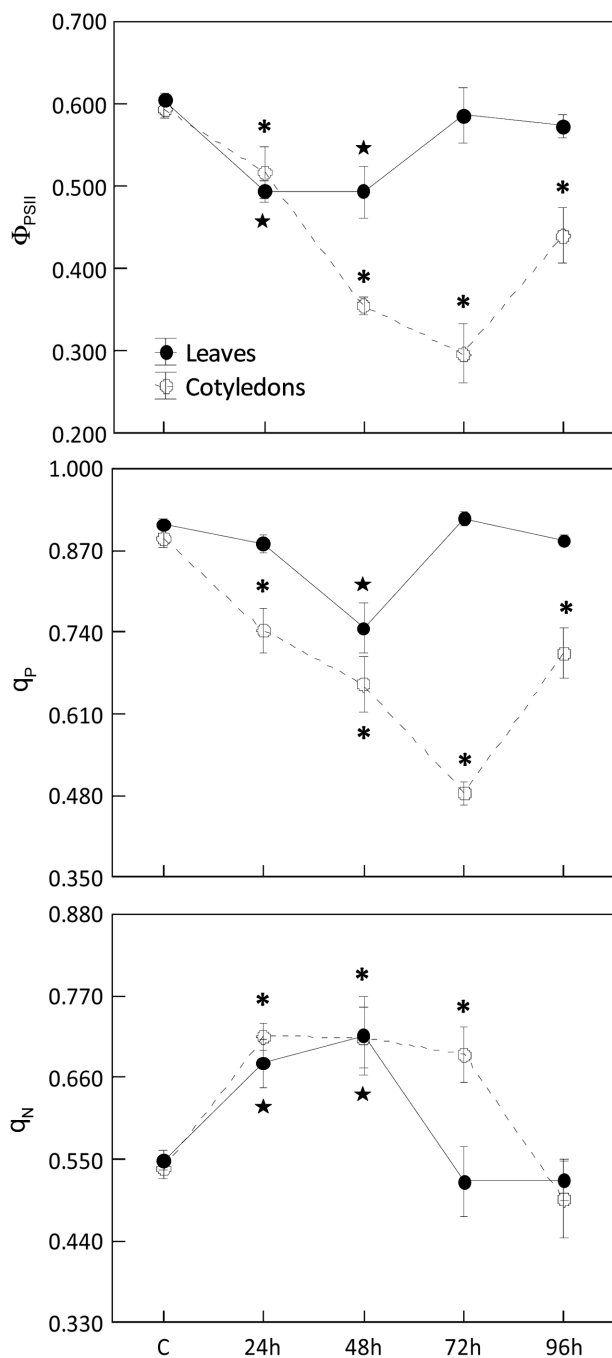
Fig. 2. Changes in Φ_{PSII} (PSII quantum efficiency), q_P (photochemical quenching coefficient) and q_N (nonphotochemical quenching coefficient) in leaves and cotyledons observed 24, 48, 72 and 96 h after inoculation. Points represent means ± standard error. C – means for total measurements performed in parallel 24, 48, 72 and 96 h after water inoculation of control plants. For cotyledons *P < 0.05 vs. control, Student's t-test. For leaves ★P < 0.05 vs. control, Student's t-test.
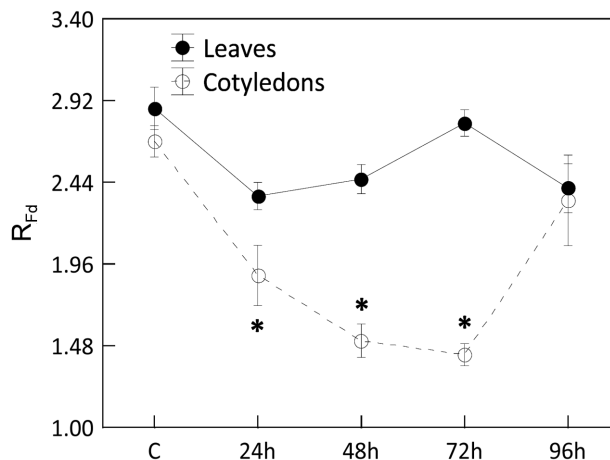

Fig. 3. Changes in R_{fd} (vitality index of the photosynthetic apparatus) in leaves and cotyledons observed 24, 48, 72 and 96 h after inoculation. Points represent means ± standard error. C – means for total measurements performed in parallel 24, 48, 72 and 96 h after water inoculation of control plants. For cotyledons *P < 0.05 vs. control, Student's t-test. For leaves ★P < 0.05 vs. control, Student's t-test.

not inoculated with *P. lingam* spores was similar at 24, 48, 72 and 96 h; that is why these changes are presented as single means.

DISCUSSION

Phoma lingam infection reduces winter rape yields (West et al., 2001; Jędryczka, 2007). The extent of the damage depends in part on the resistance of the photosynthetic apparatus during a pathogen attack. Chlorophyll fluorescence technique enables estimation of the photosynthetic efficiency of the PSII system under environmental stresses (Lichtenthaler and Miehé, 1997). Measurement from chlorophyll fluorescence imaging is a noninvasive method which gives reliable results useful in characterizing the activity of the photosynthetic apparatus across the whole leaf surface (Ning et al., 1995, 2000a).

The photosynthetic apparatus of cotyledons and leaves of oilseed winter rape exhibited a negative response to *Phoma lingam* inoculation. The effect disappeared faster in leaves than in cotyledons. Disturbed photosynthetic apparatus activity under *Phoma lingam* infection was reflected in decreased photochemical efficiency of PSII (F_v/F_m) (Fig. 1), low efficiency of excitation energy capture by PSII centers, and low efficiency of light trapping by PSII light-harvesting antennae (F'_v/F'_m) (Fig. 1). The infected plants also showed decreased accumulation of unreduced primary electron acceptors (Q_A) of photosystem II, which accept excitation energy in order to
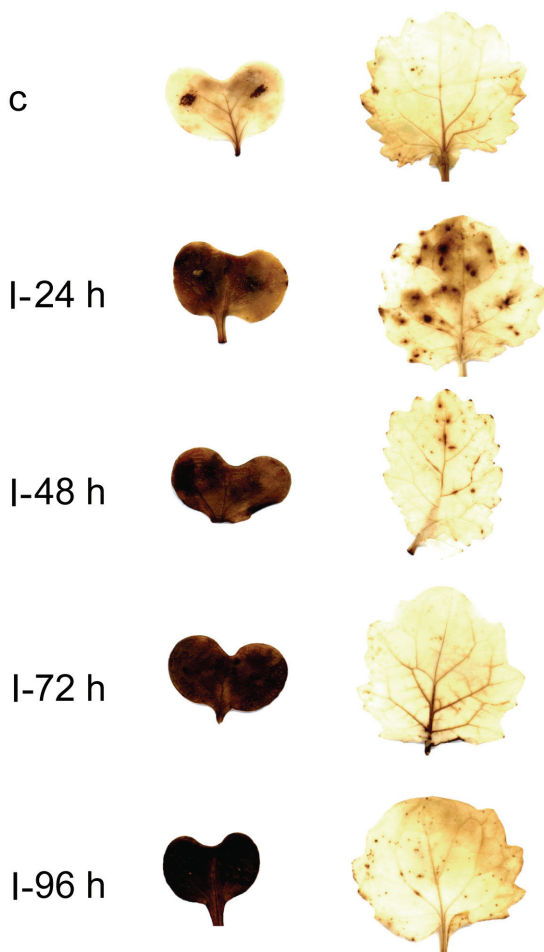
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Fig. 4. Histochemical detection of H_2O_2 accumulation in cotyledons and leaves at 24, 48, 72 and 96 h after inoculation. C – control leaves and cotyledons; I – cotyledons and leaves inoculated with *P. lingam* spores.

Fig. 5. Changes in β-1,3-glucanase and chitinase activity in leaves observed 24, 48, 72 and 96 h after inoculation. Points represent means ± standard error. C – means for total measurements performed in parallel 24, 48, 72 and 96 h after water inoculation of control plants. For leaves *P < 0.05 vs. control, Student's t-test.

pass it on to other photochemical processes (q_P) (Fig. 2) (Bolhar-Nordenkampf and Öquist, 1993). Another symptom indicating disturbed photosynthetic apparatus activity was linked to low efficiency of excitation energy utilization by chlorophyll *a* in the process of photosynthesis (Φ_{PSII}) (Fig. 2) (Maxwell and Johnson, 2000). In our experiment the high values of q_N, related to energy emitted as heat (Baker and Rosenqvist, 2004), were correlated with perturbations in photosynthetic apparatus activity in both leaves and cotyledons inoculated with *Phoma lingam*. Other experiments have shown the usefulness of chlorophyll fluorescence imaging in plant physiology for identifying plant-pathogen interactions and stress localization (Scholes and Rolfe, 1996; Nedbal et al., 2000a,b; Soukupová et al., 2003; Chaerle et al., 2004; Berger et al., 2007).
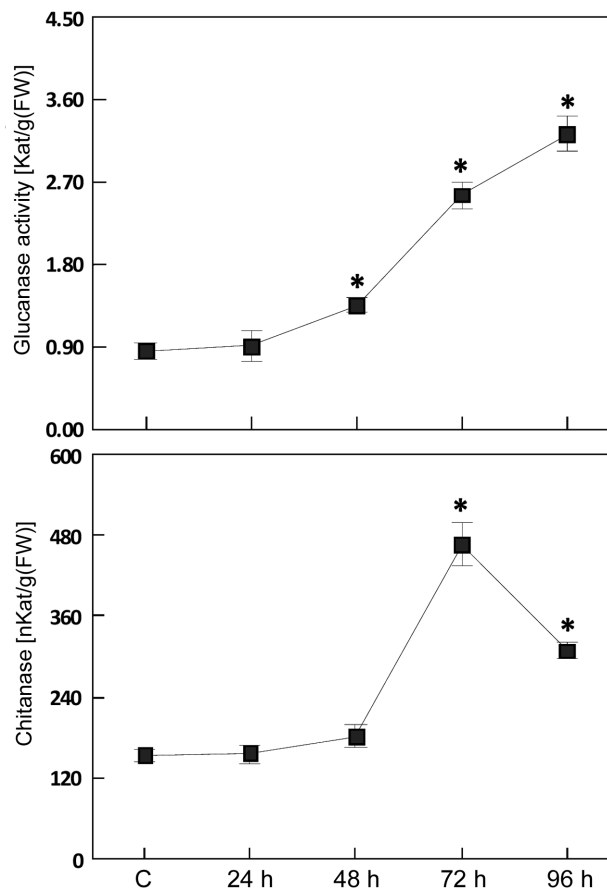
Four days after inoculating cauliflower leaves with *Phoma lingam* spores, Lai et al. (2011) detected changes in chlorophyll fluorescence parameters (e.g., F_v/F_m, Φ_{PSII}, q_P, q_N, ETR) before any signs were visible on leaves.

In the cotyledons the reaction of the photosynthetic apparatus was stronger and lasted longer, probably due to direct inoculation; light energy capture and transfer in the photochemical phase of photosynthesis reduced was more than in the indirectly inoculated leaves (Figs. 1, 2). The lower values of all chlorophyll fluorescence parameters except q_N in cotyledons can also be explained by mechanical stress (Quilliam et al., 2006). Puncturing cotyledons with a needle might enhance the effect of inoculation by generating additional free radicals. That might also help explain the slower recovery to control lev-

els in cotyledons. Recovery of chlorophyll fluorescence parameters to control levels, observed in cotyledons at 96 h, probably after inoculation, was connected with pathogen-induced changes in cell metabolism, including de novo synthesis of photosynthetic products. Elevated values of chlorophyll fluorescence parameters at inoculation sites have been reported by other authors (Lai et al., 2011; Pomar et al., 2004). In the indirectly inoculated leaves the initial disturbances of photosynthetic apparatus activity might be attributed to the increased H_2O_2 content observed at 24 h (Fig. 4). We suggest that the higher hydrogen peroxide concentration in the infected leaves should be considered a biochemical factor inducing a systemic reaction in the form of enhanced chitinase and β-1,3-glucanase activity (Fig. 5) rather than a sign of the formation of new infection foci. Anand et al. (2003) demonstrated the role of PR proteins, including chitinase and β-1,3-glucanase, in defense mechanisms against fungal pathogen infection.

In this work we used chlorophyll fluorescence to detect early disturbance of photosynthetic apparatus activity in oilseed winter rape inoculated with *Phoma lingam* spores. We demonstrated that those disturbances can be detected as soon as 24 h after inoculation in directly inoculated cotyledons and indirectly inoculated leaves. The changes may be connected with increased concentrations of hydrogen peroxide, an element of the hypersensitive response in cotyledons and which induced accumulation of PR proteins in leaves. Chlorophyll fluorescence measurements offer a promising tool for selecting genotypes resistant to blackleg caused by the fungus *Leptosphaeria maculans*. Achievement of that goal will require further experiments and testing of more oilseed winter genotypes differing in resistance to *Phoma lingam* infection.

AUTHORS' CONTRIBUTIONS

KH study conception and design; KH acquisition of data; KH, TH, MG and MR analysis and measurements; KH interpretation of data; KH drafting of manuscript; MR critical revision of manuscript. The authors declare that there are no conflicts of interests.

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