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Influence of nitric oxide and reactive oxygen species on development of lettuce downy mildew in *Lactuca* spp.

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Abstract The role of nitric oxide and reactive oxygen species, molecules indispensable for plant-pathogen signalling, was studied in the *Lactuca* spp.-*Bremia lactucae* pathosystem. Using a leaf disc model the translaminar effect of various compounds affecting their metabolism was studied by light microscopy. Time course studies revealed a slowdown in the development of *B. lactucae* (race BL16) infection structures by rutin (scavenger of reactive nitrogen and oxygen species) and SNP (NO donor) within 48 h post inoculation, followed by a retardation of sporulation. Application of the specific NO scavenger, PTIO, accelerated penetration of *B. lactucae* but had no further effects on the plant-pathogen interaction. Inhibitors of NO synthase (L-NAME) and nitrate reductase (sodium tungstate) did not influence pathogen development. Our results suggest that drastic change in the NO: hydrogen peroxide ratio seems to determine the pathogen's fate. NO synthase-like activity significantly increased early after *B. lactucae* challenge in resistant *L. virosa*. Confocal laser scanning microscopy revealed the accumulation of nitric oxide in the penetrated cells, pointing to a role in the initiation of the hypersensitive reaction. The tips of germ tubes and appressoria of *B. lactucae* also accumulated NO, suggesting an essential role for this molecule in penetration of the biotrophic pathogen. Additionally, temporal changes in endogenous levels of rutin and quercetin in extracts from *Lactuca* spp. leaves will be discussed in connection to their role as part of the antioxidative machinery that influences the plants' susceptibility/resistance to lettuce downy mildew.

Keywords Antioxidants · *Bremia lactucae* · *Lactuca sativa* · *Lactuca saligna* · *Lactuca virosa* · Oomycete · Oxidative stress · Quercetin · Rutin · Sodium nitroprusside

Abbreviations

DAF-FM DA	4-amino-5-(N-methylamino)-2′, 7′-difluorofluorescein diacetate
hpi	hours post inoculation
HR	hypersensitive response
L-NAME	N^G-nitro-L-arginine-methylester
NOS	nitric oxide synthase
NR	nitrate reductase
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

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PV	primary vesicle
ROS	reactive oxygen species
RNS	reactive nitrogen species
SNP	sodium nitroprusside
SD	standard deviation
SV	secondary vesicle

Introduction

Throughout their co-evolution, plants and pathogens have continually confronted each other in a struggle for growth and survival. Numerous components involved in pathogen perception, signal generation and transmission, and activation of defence responses have already been identified whilst others wait to be understood. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) rank among the crucial components of signalling pathways in stressed plants (Delledonne et al. 2001, 2002; De Gara et al. 2003; Wendehenne et al. 2004; Yoshioka et al. 2009; Zaninotto et al. 2006).

Nitric oxide (NO) is an essential regulatory molecule of plant immunity in synergy with reactive oxygen species (Arasimowicz and Floryszak-Wieczorek 2007). Induction of local biochemical defence responses in plant cells surrounding the infection site correlates with the accumulation of RNS. NO as a significant component of signalling networks is able to diffuse through membranes thanks to its hydrophobicity and small size (De Gara et al. 2003). The production of NO in plants was shown to be catalyzed by nitrate reductase (EC 1.7.1.1.) and nitric oxide synthase-like enzyme(s). However, the identity and structure of plant NO synthase has not been elucidated so far (Corpas et al. 2009; Wilson et al. 2008). NO can be also generated in plant cells by non-enzymatic processes like NO_2^- dismutation or reduction (Cooney et al. 1994; Stöhr and Ullrich 2002; Yamasaki 2000). In interactions with fungal pathogens *sensu lato* ROS and RNS are required for the initial signalling between both partners as well as for the activation of plant defence. Local generation of ROS is a typical feature of fungal pathogenesis, related to the active penetration of host cells (Sedlářová et al. 2007; Tománková et al. 2006). In resistant plants ROS and RNS modulate gene expression, induce structural defences or a local cell death, i.e. hypersensitive reaction (HR). Since the HR is accompanied by the generation of excess amounts of oxidative molecules, the fate of cells surrounding the infection site is determined by their ability to buffer this oxidative stress (Mur et al. 2008). Host cell collapse generally coincides with cessation of the attempted colonization, thus biotrophic pathogens must avoid triggering this process by, for example, elicitors released during cell wall penetration (Panstruga 2003). Additionally, ROS have direct antimicrobial effects (Shetty et al. 2008). The strategy of biotrophs may involve reduction of oxidative stress in the penetrated cell, e.g. by peroxidase production in the infection structures (Sedlářová et al. 2007). Antioxidant mechanisms involve specialized enzymes (superoxide dismutase (EC 1.15.1.1), peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6)), as well as non-enzymatic systems (ascorbate, glutathion, α-tocoferol, β-caroten, phenolic compounds) (Blokhina et al. 2003; De Gara et al. 2003; Grace 2005).

Lettuce and its wild relative *Lactuca* spp. are threatened by *Bremia lactucae* infection that causes downy mildew with enormous economic losses in crop yield (Lebeda et al. 2002). Due to its high genotypic and phenotypic variability, this pathosystem is a widely used model to study plant-biotrophic oomycete interactions from the field to molecular level (Lebeda et al. 2008a, b). The role of ROS and antioxidative enzymes in these interactions has been already shown (Sedlářová et al. 2007). An intensive accumulation of H_2O_2 early after inoculation was linked to race-specific resistance by initiation of HR, although variations in timing and intensity were found among genotypes. The tissues surrounding the HR showed an induction in antioxidative capacity, mainly due to an increased POX activity. This allowed the confinement of the HR cells with their excess in ROS. H_2O_2 and POX were also observed in *B. lactucae* infection structures during initial stages of the infection process (Sedlářová et al. 2007). Recently, accumulation of NO in germ tubes, appressoria and penetration pegs of powdery mildew, was proposed as an important determinant for successful invasion into host tissues (Piterková et al. 2009; Prats et al. 2008).

Our recent work describes the role of NO in the development of infection structures of downy mildew, *Bremia lactucae*, as well as in interacting host cells of *Lactuca* spp. The effect of external application of compounds modulating NO levels was studied in a series of experiments involving four *Lactuca* spp.-*Bremia lactucae* interactions with differential phenotype of susceptibility or resistance.

Materials and methods

Pathogen isolate

The experiments were performed with *Bremia lactucae* Regel race BL 16 (sextet code EU-A63/31/02/00) from the collection of the Department of Botany, Palacký University in Olomouc, included in the Czech National Collection of Microorganisms (collection number UPOC-FUN-013). The isolate was maintained and multiplied on seedlings of susceptible lettuce, *L. sativa* (cv. Cobham Green or cv. British Hilde), grown as reported previously (Sedlářová et al. 2001, 2007).

Plant material and cultivation

Eight-week-old plants of *Lactuca* spp. genotypes with different phenotypes of response to *B. lactucae* (race BL16) were used: susceptible *L. sativa* L. (UCDM2) and resistant *L. sativa* L. (Mariska), *L. saligna* L. (CGN 05271) and *L. virosa* L. (NVRS 10.001 602). Histology of these plant genotype-pathogen race interactions has been described previously (Sedlářová et al. 2007). Plants were grown in a soil/peat (2:1, *v*/*v*) mixture in a growth chamber at 18/15°C (day/night), 12 h photoperiod, irradiance of 100 $\mu E.m^{-2}.s^{-1}$ provided by warm-white fluorescent lamps. Following inoculation, the cultivation temperature was decreased to 15/10°C and the plants were kept in dark during first 24 h to establish optimal conditions for pathogen development.

Inoculation, incubation and collection of samples

Whole plants/leaf discs were inoculated by spraying with a suspension of *B. lactucae* conidia in distilled water (approx. 5×10^5 ml^{-1}) and further incubation followed previously described procedures (Sedlářová et al. 2007). The 4th to 6th youngest leaves per plant were harvested 0, 4, 8, 24, 48, 72, 96, 168 or 216 h post inoculation (hpi), and either subjected to histochemical staining followed by confocal microscopy or frozen in liquid nitrogen and stored at −80°C until enzyme activity or concentration of antioxidants was studied in the leaf extract.

For light microscopy experiments, leaf discs (12 mm in diameter), derived from 4th–6th youngest leaf of a plant, were placed abaxial side up on wet filter paper in Petri dishes and subsequently inoculated.

Modulation of NO levels

Most of the following chemicals were purchased from Sigma-Aldrich, Czech Republic, except the glycerol (Tamda, Czech Republic) and NO-specific probe (Axxora, U.S.A.).

Different NO level modulators were applied to the filter papers under the leaf tissues in Petri dishes. The filters were saturated with 10 ml of the solution to be tested. The final concentrations of the studied substances were 0.1 mM SNP (NO donor), 0.1 mM PTIO (NO scavenger), 0.1 mM rutin (NO/ROS scavenger), 1 mM L-NAME (NOS inhibitor), and 5 mM Na_2WO_4 (inhibitor of nitrate reductase). In case of enzyme inhibitors, the tissues were pre-incubated 1 h in vacuum prior to the inoculation, other variants were inoculated directly. The control leaf discs were incubated with deionised water. Leaf discs were collected 4, 8, 24 and 48 hpi. Each Petri dish contained five leaf discs from one plant for each time interval and three Petri dishes per solution variant were used, i.e. each sample contained 15 leaf discs.

Light microscopy of *B. lactucae* development and *Lactuca* spp. tissue reactions

Leaf discs were fixed in 100% acetic acid for 2 days and then transferred and stored in 85% glycerol. Prior to observation the specimens were stained with 1% Aniline Blue (*w*/*v*). Pathogen development and host tissue reactions were examined by light microscopy (Olympus BX-60) and documented with a CCD camera (Olympus DP70; Olympus C&S, Prague, Czech Republic). In each variant five leaf discs and 15 spores/infection sites per leaf disc were randomly evaluated. The results were expressed as a mean frequency and standard deviation for each substance and time interval.

Statistical analysis

For each genotype the effect of the treatment on pathogen development and tissue response was statistically analyzed using NCSS 2001 software (Kaysville, UT, U.S.A.). The frequency of infection structures or hypersensitive cells was analyzed by ANOVA applying the Tukey-Kramer multiple-comparison test. Significantly different mean values (at 0.05 level) were marked by asterisks in Figs. 2, 3, 4 and 5.

Localisation of NO production by confocal microscopy

Small pieces of *Lactuca* spp. leaf tissues were cut off plants or leaf discs incubated with either water or modulators of NO production, incubated in 20 μM solution of DAF-FM DA (4-amino-5-(N-methylamino)-2′,7′-difluorofluorescein diacetate) (Axxora, U.S.A.) for 30 min, washed with buffer and mounted in 20 mM K-phosphate buffer (pH 7.0) on microscopic slides and subjected to confocal laser scanning microscopy (Olympus Fluorview 1000 attached to an inverted microscope IX81). Excitation was provided by the 488 nm line of an argon ion laser; a 505-nm dichroic filter and 519-nm longpass emission filter were applied to detect the signal. The base intensity of lasers was set according to samples from uninoculated plants at the beginning of each experiment. Negative controls were pre-treated with NO scavenger (0.1 mM c-PTIO) or NO synthase inhibitor (10 mM L-NAME) for 30 min prior to staining. The positive control was pre-incubated with a NO generator (0.1 mM SNP) (Piterková et al. 2009).

Preparation of plant extracts

Frozen leaf tissues were homogenized (1:2; *w*/*v*) with ice-cold 20 mM HEPES buffer, (pH 7.5), containing 2 mM ethylenediamine tetraacetic acid (EDTA), 0.5% polyvinylpolypyrrolidone (PVPP) and 10% glycerol in a chilled mortar. Prior to homogenisation, 5 mM dithiothreitol (DTT), 2 mM aprotonin and 2 mM phenylmethylsulphonyl fluoride (PMSF) were added to the extraction HEPES buffer. Leaf homogenates were centrifuged (16,000 × *g*, 15 min, 4°C) and supernatants immediately used for the determination of NO production.

Determination of NOS-like activity

NOS-like activity in plant extracts was determined spectrophotometrically by an oxyhemoglobin method based on the reaction of NO with oxyhemoglobin to yield methemoglobin and nitrate (Hevel and Marletta 1994) as previously described in tomato (Piterková et al. 2009). The reaction medium contained 10 μM oxyhemoglobin, 1 mM L-Arg, 1 mM $CaCl_2$, 1 mM Mg^{2+}, 1 μM calmodulin, 140 μM NADPH, 1 μM FAD, 1 μM FMN and 10 μM tetrahydrobiopterin in 100 mM HEPES buffer, pH 7.5. Norvaline as a competitive inhibitor of potentially interfering arginase was added to give a final 1 mM concentration. Superoxide dismutase (50 U) and catalase (100 U) were added to prevent interference by $O_2^{\cdot-}$ and/or H_2O_2. All measurements were performed at 37°C and the absorbance was determined at λ=401 nm on a microplate reader Synergy (Biotek, U.S.A.). The blank values were subtracted from all measured values (leaf extracts replaced by the same volume of buffer in the reaction mixture). For each variant, three measurements for each of the above mentioned three independent sets of samples were conducted, i.e. nine values were obtained. Data are presented as mean ± standard deviation (SD).

Rutin and quercetin content in leaves

Leaf samples were frozen in liquid nitrogen and lyophilised. Dried leaf samples were pulverized in a chilled mortar and transferred to plastic microtubes. Samples were extracted with 2 ml of 70% methanol by sonication (5 min) and incubation for 1h, followed by centrifugation (13,000 × *g*, 10 min, RT). Supernatants of methanol extracts were transferred to clean microtubes and evaporated at 40°C under a nitrogen stream. Samples were analysed by HPLC (system Gold; Beckman, U.S.A.) on HS PEG column (150× 4 mm, 5 μm, Supelco, U.S.A.), using isocratic elution with the mobile phase 50% methanol in 0.1 M formic acid at 30°C. The retention time of rutin and quercetin was 2.1 and 6.8 min, respectively. The amount of rutin and quercetin in leaf extracts was quantified using a calibration set of commercial rutin and quercetin standards. Results were expressed as molar concentration per dry weight and represent mean of 9 measurements and standard deviation.

Results

Development of *B. lactucae* infection structures and reaction of *Lactuca* spp. tissues

Germination and development of *Bremia lactucae* (race BL16) primary infection structures was studied on leaf discs incubated with solutions of compounds that influenced NO concentration within host tissues (Fig. 1). Pathogen spores usually germinate and form appressoria few hours after inoculation (Fig. 1a, b).

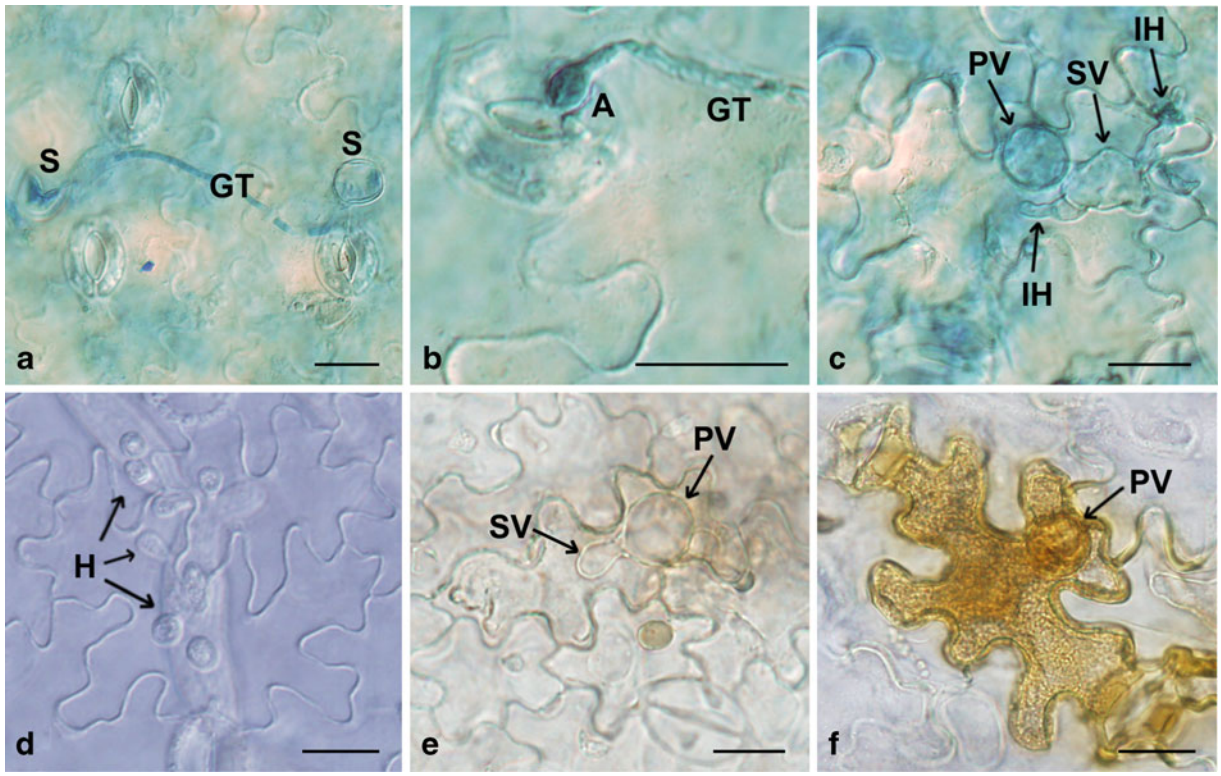

Fig. 1 Infection structures of *Bremia lactucae* (race BL16) and response of *Lactuca* spp. tissues. **a** Germination. **b** Detail of germ tube with appressorium on *L. virosa* (NVRS 10.001 602) 24 hpi. **c, d** Epidermal cells of susceptible *L. sativa* (UCDM2) penetrated by: **c,** primary infection structures 24 hpi and **d,** haustoria 168 hpi. **e** Onset of hypersensitive reaction in *L. saligna* (CGN 05271) 48 hpi. **f** Late stage of hypersensitive reaction in *L. virosa* (NVRS 10.001 602) 48 hpi. Infection structures: spore (*S*), germ tube (*GT*), appressorium (*A*), primary vesicle (*PV*), secondary vesicle (*SV*), intracellular hypha (*IH*), haustorium (*H*). The bar corresponds to 20 μm

Penetration of epidermal cell is followed by development of primary and secondary vesicles (Fig. 1c, e) and either 1/ further intercellular growth with abundant haustoria formation in susceptible host tissues (Fig. 1d); or 2/ cessation of pathogen growth in resistant plants often followed by hypersensitive reaction (Fig. 1f).

Pathogen germination on susceptible *L. sativa* (UCDM2) and resistant *L. sativa* (Mariska), *L. saligna* (CGN 05271) and *L. virosa* (NVRS 10.001 602) was recorded at 4 and 8 hpi (Fig. 2). Application of the NO donor SNP reduced conidial germination. Rutin had an even stronger inhibitory effect on germ tube formation in all genotypes, namely 4 hpi. Similarly, these compounds reduced the penetration rate in all genotypes from 8 hpi on (data not shown). The application of rutin had the opposite effect on *L. saligna*, where it intensified *B. lactucae* penetration 24 hpi (Fig. 3). The frequency of primary vesicles was increased in PTIO (a specific NO scavenger)-treated tissues, in all genotypes from 24 hpi (Fig. 3). The NOS inhibitor L-NAME and the NR inhibitor sodium tungstate had no significant effect on *B. lactucae* germination and primary vesicle development (Figs. 2 and 3).

Development of secondary vesicles was not affected by PTIO; all other compounds delayed growth and decreased frequency of secondary vesicles, especially in *L. sativa* genotypes (Fig. 4). The strongest effect was recorded for rutin and SNP both 24 and 48 hpi. Additionally, variation in expression of HR (Fig. 1e, f) was evaluated by light microscopy in all samples. Significant differences were recorded only for *L. virosa* (NVRS 10.001 602) 48 hpi, as presented in Fig. 5. The proportion of infection sites with hypersensitively responding cells

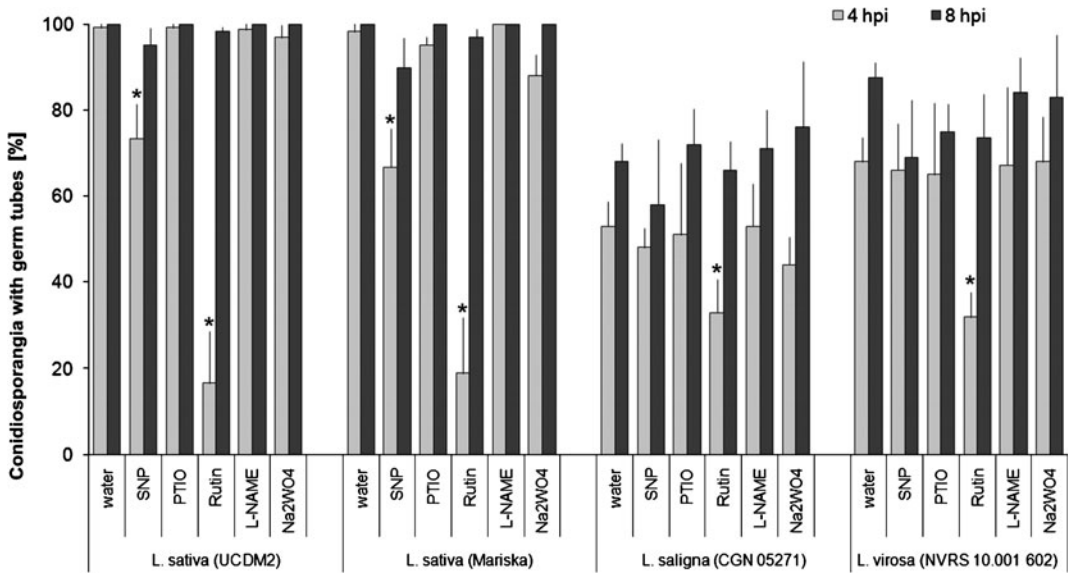

Fig. 2 Germination of *B. lactucae* (race BL16) conidiosporangia on leaf discs of *Lactuca sativa* (UCDM2, and Mariska), *L. saligna* (CGN 05271) and *L. virosa* (NVRS 10.001 602) incubated on distilled water (control) and solutions of substances modulating RNOS level (SNP, PTIO, rutin, L-NAME and sodium tungstate) 4 and 8 hpi. Data are given as mean ± SD, $n=15$

(Fig. 5a) was significantly lowered by application of SNP, PTIO, L-NAME and strongest by rutin. The average number of cells per HR (Fig. 5b) was increased by the enzyme inhibitors.

Histochemical localization of NO in inoculated leaves

Localization of nitric oxide within tissues of *Lactuca* spp. and infection structures of *Bremia lactucae* (race

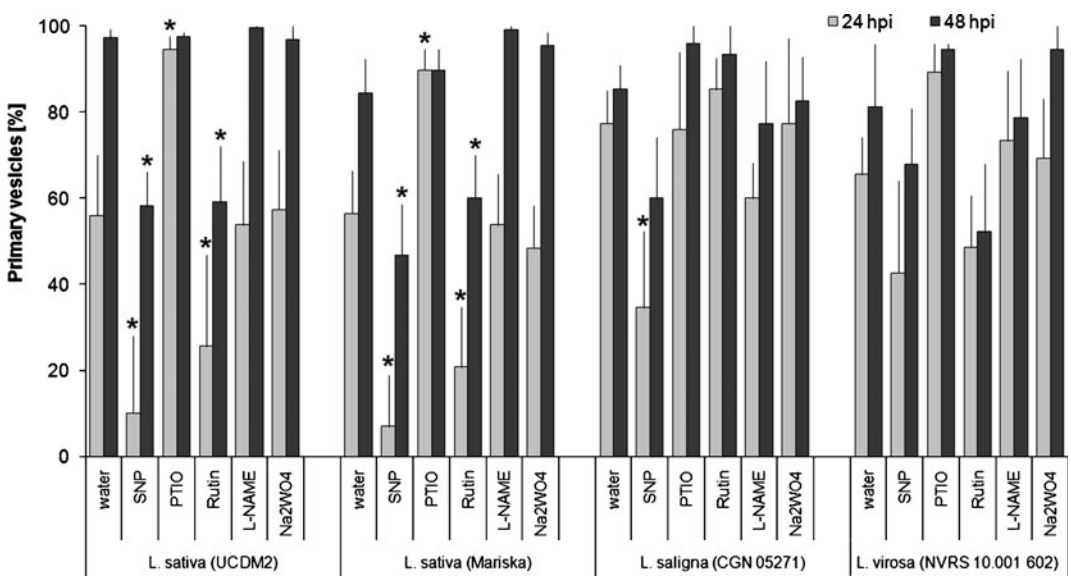

Fig. 3 Penetration rate of *B. lactucae* (race BL16), expressed as percentage of epidermal cells in contact with pathogen that contained primary vesicles. Leaf discs of *Lactuca sativa* (UCDM2, and Mariska), *L. saligna* (CGN 05271) and *L. virosa* (NVRS 10.001 602) incubated on distilled water (control) and solutions of substances modulating RNOS level (SNP, PTIO, rutin, L-NAME and sodium tungstate) are compared 24 and 48 hpi. Data are given as mean ± SD, $n=15$

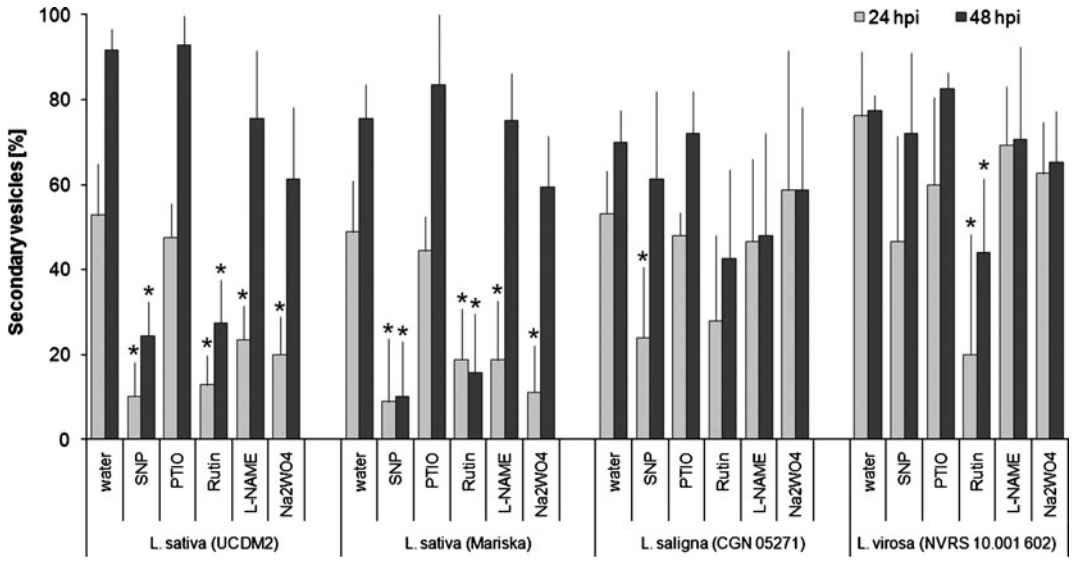

Fig. 4 Proportion of epidermal cells with developed secondary vesicle of *B. lactucae* (race BL16). Data recorded for leaf discs of *Lactuca sativa* (UCDM2, and Mariska), *L. saligna* (CGN 05271) and *L. virosa* (NVRS 10.001 602) incubated on distilled water (control) and solutions of substances modulating RNOS level (SNP, PTIO, rutin, L-NAME and sodium tungstate) are compared 24 and 48 hpi. Data are given as mean ± SD, n=15

BL16) was studied by confocal laser scanning microscopy following staining with the fluorescent probe DAF-FM DA (Fig. 6). Production of NO was observed in the infection structures of *B. lactucae* grown both on susceptible and resistant genotypes, although these differed in timing of the pathogen development. An intensive signal in the tip of the germ tube and appressorium (Fig. 6a) indicates an essential role of the molecule in the process of pathogen infection structures growth and penetration. A weaker NO signal was detected also in developing primary and secondary vesicles (Fig. 6f), intracellular hyphae and in haustoria on susceptible *L. sativa* (UCDM2) (Fig. 6d). In *L. virosa* (NVRS 10.001 602), a genotype with an abundant hypersensitive response, NO was frequently localized in penetrated cells undergoing HR before occurrence of detectable necrosis (Fig. 6b, c).

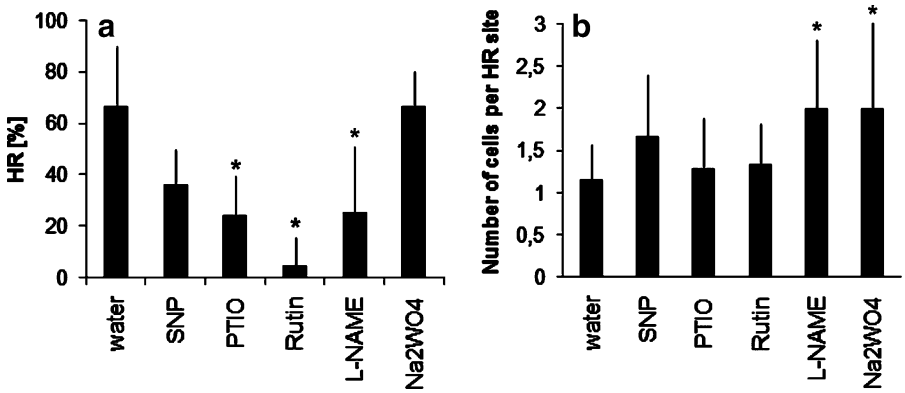

Fig. 5 *L. virosa* (NVRS 10.001 602)-*B. lactucae* (race BL16) interaction 48 hpi: **a** proportion of infection sites with hypersensitive response of epidermal cells; **b** average number of cells per HR. Leaf discs were incubated on distilled water (control) and solutions of substances modulating RNOS level (SNP, PTIO, rutin, L-NAME and sodium tungstate). Data are given as mean ± SD, n=45

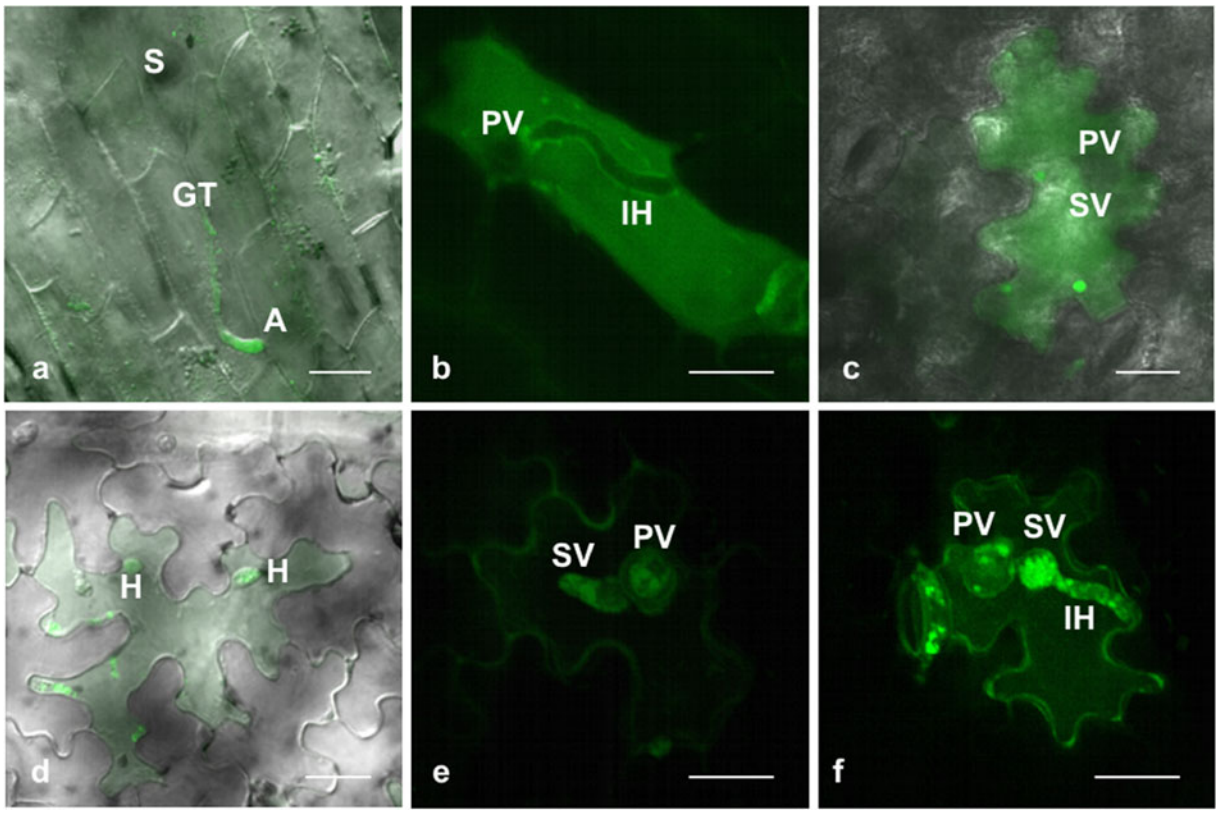

Fig. 6 Nitric oxide production in lettuce downy mildew and challenged host epidermal cells visualized by confocal laser scanning microscopy. **a** During germination the signal for NO was detected in *B. lactucae* germ tubes and appressoria, *L. virosa* (NVRS 10.001 602) 24 hpi. **b, c** In resistant genotypes NO accumulates in pre-necrotic cells 24 hpi: **b,** *L. saligna* (CGN 05271); **c,** *L. virosa* (NVRS 10.001 602). **d** Interestingly, signal for NO was found in epidermal cells of susceptible *L. sativa* (UCDM2) stressed by formation of pathogen haustoria 168 hpi. **e** At late stages of infection the autofluorescence of infection structures within HR co-localized with **f,** a signal of NO-sensitive fluorescent dye DAF-FM DA, *L. virosa* (NVRS 10.001 602) 168 hpi. Infection structures: spore (*S*), germ tube (*GT*), appressorium (*A*), primary vesicle (*PV*), secondary vesicle (*SV*), intracellular hypha (*IH*), haustorium (*H*). Bar corresponds to 20 μm

Changes of NOS-like activity in leaves inoculated by *B. lactucae*

NOS-like activity was determined as L-arginine and NADPH-dependent NO production by the oxyhemoglobine method in extracts of 4th–6th youngest plant leaves during 216 hpi (Fig. 7). Four *Lactuca* spp. genotypes involved in our study were characterized by differential basal levels of NOS-like activity. The highest activity was found in non-infected plants of the resistant genotypes of *L. virosa* (NVRS 10.001 602) and *L. saligna* (CGN 05271), whereas almost no activity was detected in *L. sativa* genotypes 0 hpi (Fig. 7). Following inoculation, a rapid increase in NOS-like activity was recorded in *L. virosa* (NVRS 10.001 602), to 300% of constitutive level at 4 and 8 hpi. The activity rapidly decreased from 24 hpi to the level of uninoculated plants. The resistant *L. sativa* (Mariska) displayed an increased activity of NOS-like enzyme upon inoculation with a maximum between 48 and 168 hpi. On the other hand, the NOS-like activity in resistant *L. saligna* (CGN 05271) with reduced hypersensitivity moderately increased at 4 hpi but was lower again at later stages of the infection process. Minor fluctuation of NOS-like activity characterized also susceptible *L. sativa* (UCDM2), with moderate increase between 4–24 hpi and 72–216 hpi.

Rutin and quercetin content in leaves

Contents of rutin and its aglycone form, flavonol quercetin, were determined by HPLC analysis in leaf extracts of *Lactuca* spp. plants during 216 h following

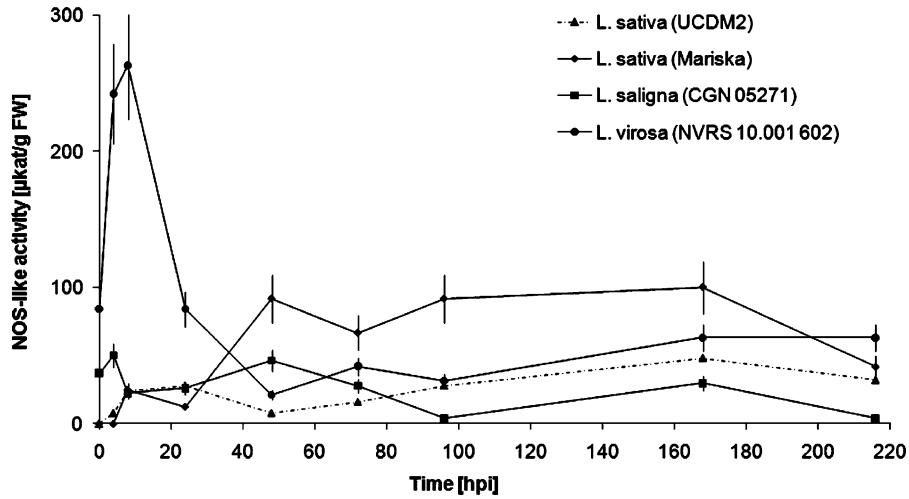

Fig. 7 Changes in NOS-like activity in leaves of *Lactuca* spp. plants infected with *B. lactucae* (BL 16) during 216 hpi. Data are given as mean ± SD, n=9

inoculation by *B. lactucae*. The highest level of rutin and quercetin, 2.4 and 7.5 mmol/g FW, respectively, was detected in leaf tissues of *L. virosa* (Fig. 8). Rutin content decreased 24 hpi, however, increased during 48–216 hpi. Susceptible *L. sativa* (UCDM2) was characterized by rather low constitutive level of both compounds which increased at late stages of infection, 216 hpi. During pathogenesis of *B. lactucae* on resistant *L. sativa* (Mariska) the content of both substances gradually decreased to approx. 50%

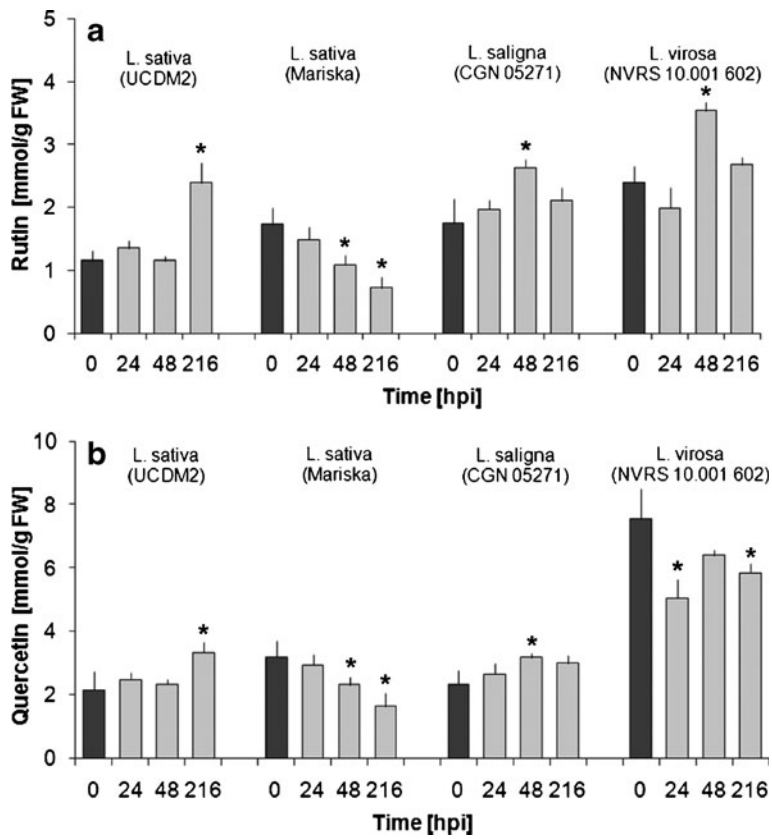

Fig. 8 Content of rutin (**a**) and quercetin (**b**) in leaf extracts from plants infected with *B. lactucae* (BL 16) (grey column) during 216 hpi. Controls are non-infected plants 0 hpi (black column). Data are given as mean ± SD, n=9

amount at 216 hpi. Intermediate levels of rutin and quercetin were found in intact tissues of *L. saligna* (CGN 05271) which increased in infected plants starting at 24 hpi and peaking at 48 hpi (Fig. 8).

Discussion

Histological and biochemical features of the *Lactuca* spp.-*Bremia lactucae* interaction, a model plant-biotrophic oomycete pathosystems, have been studied intensively in our laboratory for two decades (Lebeda et al. 2001, 2002, 2008b). It is a well accepted fact that spore germination of the pathogen isolates reaches the highest rate on genotypes of the original host plant species (e.g. Sedlářová et al. 2001). Thus, in the present study almost 100% of the conidiosporangia of *B. lactucae* (race BL16) germinated on lettuce leaf tissues (cvs. UCDM2 and Mariska), whereas on wild *Lactuca* spp. plants, *L. saligna* (CGN 05271) and *L. virosa* (NVRS 10.001 602), the number was reduced to approx. 60%. *B. lactucae* germ tubes also had a reduced length on the wild *Lactuca* spp. compared to *L. sativa* genotypes and the interspecific breeding lines (Lebeda and Pink 1998), and significant differences in the length of germ tubes between seedlings and adult plants of *L. sativa* were described (Lebeda and Reinink 1991). This can be explained by leaf surface characteristics (composition of waxes, abundance of trichomes) that modify attachment of spores and shorten water availability for their germination (Lebeda et al. 2001).

Although oxidative processes are considered to be vital for plant resistance, including *Lactuca* spp. (reviewed in Lebeda et al. 2008b), the importance of NO and ROS for oomycete pathogenesis has not been reported in detail yet. In previous work (Petřivalský et al. 2007) we found that the processes localized outside of the leaves, e.g. germination and appressoria formation, are affected by the compounds infiltrated into leaves to a limited extent. Here, we report that the number of germ tubes was significantly decreased only in tissues treated with rutin 4 hpi. During penetration, the local concentrations of NO and ROS change due to a release by both pathogen infection structures and host cells. Application of a NO donor or a strong antioxidant (rutin) reduced the development of primary as well as secondary vesicles. The only exception was an increase of the downy mildew penetration rate in *L. saligna* upon rutin treatment. On the other hand, lower NO concentrations after PTIO application led to an increase in frequency of primary vesicles.

The response of the individual lettuce genotypes to *B. lactucae* (race BL16) infection was reported in a previous study (Sedlářová et al. 2001), where initiation of the HR was shown to correlate with pathogen developmental stages rather than the time-scale of pathogenesis. Here we show that application of NO level modulators mainly altered the response of plant genotypes with a pronounced HR response, i.e. *L. virosa* (NVRS 10.001 602). NO level modulators lowered the proportion of infection sites with HR but had only a minor effect on the extent of the HR (Fig. 5). The synergistic action of NO and H_2O_2 is believed to orchestrate the HR and restrict pathogen invasion (Zaninotto et al. 2006), although the interactions between NO and ROS are still a matter of discussion (Delledonne et al. 2003; Wilson et al. 2008). Interaction of NO and H_2O_2 leads to the production of either highly reactive singlet oxygen or hydroxyl radicals, both of which can induce the processes of cell death. Reaction of NO with O_2^- leads to the formation of a strong oxidant compound, peroxynitrite (ONOO–; Saito et al. 2006). Accumulation of superoxide has not been recorded in the *Lactuca* spp.-*B. lactucae* interactions, however, H_2O_2 production colocalizes with NO (Sedlářová et al. 2007). Excess amounts of antioxidant, in the form of rutin solution applied to leaf tissues, diminished the HR in *L. virosa* and concurrently retarded pathogen growth. A spectrum of methodological approaches to study NO is available, although the specificity of some is not yet clear (Miller and Chang 2007; Vandelle and Delledonne 2008). We localized NO in the infected cells of resistant *Lactuca* spp. before expression of symptoms as visualized by DAF FM-DA staining (Fig. 6b–d) which is in agreement with previous experiments based on application of the most sensitive assays (reviewed by Mur et al. 2006). This early NO burst has been reported to precede H_2O_2 generation, and in a plant-powdery mildew model it occurred about 6 h before the first visible signs of cell death (Mur et al. 2006). There are indications that NO can affect the generation of ROS, which are closely associated with the HR in plants. Zeier et al. (2004) demonstrated that the pathogen-challenged nitric oxide dioxygenase transgenic lines

of *Arabidopsis* exhibited not only reduced NO generation, but also a reduced oxidative burst (decreased H_2O_2 levels) and a blockage of phenylalanine ammonia lyase expression in incompatible interactions with *Pseudomonas syringae*. Interestingly, NO can influence apoptosis at many points and can be either pro- or anti-apoptotic. In plants, there have also been reports of NO suppressing cell death (Bethke et al. 2004). The relative concentrations of particular ROS and NO appear to be vital in the initiation or suppression in cell death. Our results confirm the need for NO in plant race-specific defence to oomycetes. However, no activation of host NO production has been found in interactions between animals and pathogenic oomycetes (Phillips et al. 2008).

Recently, a special emphasis has been put on approaches taking into account the life strategy of pathogens, because huge differences exist in pathogen biology, physiology, toxicology and the reactions of host plants (Glazebrook 2005). Nitric oxide was shown to participate not only in plant resistance to biotrophic pathogens but also to necrotrophs. NO and oxidative bursts were induced during compatible interaction of *Nicotiana benthamiana* with the necrotrophic fungal pathogen *Botrytis cinerea*. NO was shown to play a pivotal role also in basal defence against *B. cinerea* and *PR-1* gene expression in *N. benthamiana*. By contrast, ROS had a negative function in resistance, i.e. had a positive role in expansion of disease lesions during the *B. cinerea–N. benthamiana* interaction (Asai and Yoshioka 2009).

Both partners in plant-fungus interactions have been shown to produce NO (Mur et al. 2006; Prats et al. 2008), thus, we expected a similar phenomenon in oomycete pathogenesis. Our experiments showed a strong signal for NO in the apical parts of *B. lactucae* mycelium, tips of germ tubes and appressoria, which is prerequisite for penetration (Fig. 6a). In compatible interactions NO was detected also in haustoria (Fig. 6d) which indicates an involvement of NO in pathogen development. Sources of NO in oomycetes have not been studied but the presence of an enzyme similar to NOS is highly probable. Nevertheless, one methodological aspect of our work may be a matter of discussion. We are aware that we measured NOS-like activity in extracts of infected leaves covering the enzyme activity from both organisms. However, *B. lactucae* is a biotrophic endoparasite which makes its cultivation or detachment from infected tissues impossible (Lebeda et al. 2008b). Thus, we presume that application of constant inoculum concentration and standard cultivation conditions diminished the variation among genotypes. On the other hand, the influence of pathogen-derived enzyme activity might increase with increasing pathogen biomass during infection, especially in the susceptible genotype *L. sativa* (UCDM2) or the resistant *L. saligna* (CGN 05271), where *B. lactucae* (BL16) is arrested at later stages of development (Sedlářová et al. 2001). However, tissue and cell responses of non-host resistance of *L. saligna* accessions (Lebeda et al. 2001, 2002) are different from race-specific and field resistance of *L. sativa* (Lebeda and Reinink 1994). In *L. saligna* (CGN 05271) at 48 hpi significantly lower frequencies of hyphae and haustorium formation were observed, however, higher a frequency of HR in infection sites, in comparison with susceptible *L. sativa* (Lebeda and Reinink 1994). Nevertheless, there is also a large variation in formation and development of infection structures and tissue response in different accessions of *L. saligna* (Lebeda and Pink 1998; Lebeda and Reinink 1994; Lebeda et al. 2006) showing delicate differences in metabolic pathways (including ROS and RNS metabolism) in individual host genotype-pathogen race interactions (Sedlářová et al. 2007), primarily due to the genetic background of these interactions (Zhang et al. 2009).

Our results suggest that plants use multiple ways for the synthesis of NO, the critical molecule in plant-pathogen communication. Nitric oxide (NO) is a highly diffusible and short-lived physiological messenger. Despite its diffusible nature, NO modifies thiol groups of specific cysteine residues in target proteins and alters protein function via S-nitrosylation. Tyrosine nitration of proteins by NO was recently reported to be increased in susceptible but not in the resistant cultivar of sunflower following downy mildew (*Plasmopara halstedii*) infection. Therefore, post-translational protein modifications were proposed as a marker of nitrosative stress in the plant–pathogen interaction (Chaki et al. 2009).

External application of rutin had a striking impact on *B. lactucae* development so we analyzed the *in planta* concentrations among *Lactuca* spp. genotypes. Rutin, a plant flavonoid glycoside with antioxidant properties utilized in human medicine, is composed of flavonol quercetin and the disaccharide rutinose.

Rutin helps to maintain levels of the biological antioxidant reduced glutathione. Importantly, following nitrosation, both molecules may become pro-oxidants (Laughton et al. 1989). Only minor increases in levels of both rutin and quercetin in *L. saligna* tissues were recorded. Generally, there is no need to overstimulate the antioxidant machinery in this genotype characterized by reduction of downy mildew without large expression of HR (Lebeda and Pink 1998; Lebeda and Reinink 1994; Sedlářová et al. 2001). On the other hand, *L. virosa* is generally showing a high proportion of infection sites with HR (Lebeda and Pink 1998; Lebeda et al. 2006), expressed also phenotypically as tissue necrosis (Lebeda et al. 2002; Norwood et al. 1981). Tissues of this species are rich in both rutin and quercetin as there is a necessity to protect tissue surrounding HR cells. Higher levels of both antioxidants were found in the resistant cv. Mariska that decreased during the course of downy mildew infection. In the susceptible cv. UCDM2, an increase was found very late after inoculation (216 hpi), probably induced in order to buffer oxidative stress linked to *B. lactucae* intercellular growth. Our results show evidence for a dual (dose-dependent and balanced) role of NO and ROS in this host-pathogen interaction, that can be summarized as follows. In *L. virosa* (NVRS 10.001 602) high levels of rutin together with phenolic acids (Grúz and Sedlářová, unpublished), and peroxidase activity (Sedlářová et al. 2007) are important components of resistance mechanisms that buffer oxidative molecules released in relation to: 1) *B. lactucae* penetration of epidermal cells to limit formation of infection structures; 2) the onset of HR following *B. lactucae* recognition in the cytoplasm. Intensive hydrogen peroxide release in *L. virosa* leads to HR which often involves more than one cell from not only epidermal but also mesophyll tissue, in comparison to other resistant *Lactuca* spp. genotypes (Lebeda and Pink 1998; Lebeda et al. 2006; Sedlářová et al. 2001). On the other hand, still little is known about the physiology and biochemistry of resistance mechanisms of *L. saligna* to downy mildew, which is primarily characterized by limited HR (Lebeda and Reinink 1994; Lebeda et al. 2001, 2002) and minor changes in all of parameters investigated so far (Sedlářová et al. 2007; this paper). However, recent genetic investigations suggest that non-host resistance in *L. saligna* is the results of cumulative effects of many QTLs (at least 15) differentially operating at various developmental stages (Zhang et al. 2009). This is also supporting our recent biochemical data and their variation.

In many plants, resistance has been associated with a higher activity of antioxidant enzymes (peroxidase, catalase, superoxiddismutase) or antioxidant substances (Bolwell and Daudi 2009). However, there is no such direct link between any of the above-mentioned traits and susceptibility/resistance in *Lactuca* spp.-*B. lactucae*. Lettuce genotypes differ from other wild relative *Lactuca* spp. in many physiological features (Lebeda et al. 2008b). These differences demand further detailed study of specific host-pathogen interactions and various developmental stages. HR-based resistance in *L. virosa* as well as non-HR resistance in *L. saligna* are promising alternatives to be utilized in lettuce resistance breeding (Lebeda et al. 2009).

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