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Existing infection with *Rhynchosporium secalis* compromises the ability of barley to express induced resistance

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Abstract It has been suggested that if plants in the field are already induced, their ability to further enhance induced resistance might be compromised. This was examined in barley by inoculating the first two leaves with *Rhynchosporium secalis* prior to treatment of leaves three and four with an elicitor combination, followed by inoculation with *R. secalis*. The elicitor combination used consisted of acibenzolar-S-methyl, β-aminobutyric acid, and *cis*-jasmone, which was shown previously to provide higher levels of disease control in barley than any of the components used individually. The elicitor combination reduced infection by *R. secalis,* and led to an up-regulation of *PR1-b,* a marker gene for systemic acquired resistance, and increased activities of the defence-related enzymes cinnamyl alcohol dehydrogenase (CAD), peroxidase (POX), and β-1,3-glucanase. It also led to down-regulation of *LOX2,* a gene involved in biosynthesis of jasmonic acid. In plants where the first two leaves were inoculated with *R. secalis* prior to treatment of leaves three and four with elicitor, these increased defence responses did not occur, and control of *R. secalis* infection on leaves three and four was also reduced. These results suggest that, at least in young barley plants, prior infection with *R. secalis* compromises their ability to respond effectively to elicitors. The results might help to explain the relatively poor performance of induced resistance in the field, particularly in cereals, compared to plants grown under controlled conditions.

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Abbreviations

ASM	Acibenzolar-S-methyl
BABA	β-aminobutyric acid
CAD	Cinnamyl alcohol dehydrogenase
CJ	*cis*-jasmone
LOX2	Lipoxygenase 2
POX	Peroxidase
PR1-b	Pathogenesis-related gene 1-b

Introduction

To survive the onslaught of a myriad of attackers, such as microbial pathogens and herbivorous insects, plants have evolved mechanisms to perceive attack and then mount an appropriate defensive response. The initial response recognises common features of attacking organisms and converts the recognition into defensive action targeted against the specific attacker (Jones and Dangl 2006). This primary response is

complemented by a second line of defence known as induced resistance, which acts both locally and systemically within the plant, and is broad spectrum in its activity against attackers (Walters et al. 2007). Different types of induced resistance can be activated by the plant, including systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR tends to be triggered by organisms which cause restricted cell death, such as the hypersensitive response (HR), and is mediated by the salicylic acid (SA) signalling pathway (Durrant and Dong 2004; Pieterse and Van Loon 2007). In contrast, ISR is activated following colonisation of plant roots by selected strains of plant growth-promoting rhizobacteria (PGPR) and is mediated by jasmonic acid (JA) and ethylene (ET) (Pieterse and Van Loon 2007). Resistance can also be induced by a variety of agents, some of which mimic the action of natural inducers such as microbial pathogens and PGPR. Such agents include a functional analogue of SA, acibenzolar-S-methyl (ASM), which is known to induce SAR in a range of plant species, chitosan, a common polymer in the shells of crustaceans, exoskeletons of insects and cell walls of fungi, and the non-protein β-aminobutyric acid (BABA) (Ruess et al. 1996; Linden et al. 2000; Silué et al. 2002, Cohen et al. 2010). These agents are known as resistance elicitors or plant activators, and they offer the prospect of broad spectrum disease control in crops, based upon activation of the plant's existing resistance mechanisms (Walters et al. 2005).

Induced resistance can lead to the direct activation of defences, such that application of an elicitor can trigger defences even in the absence of an attacker. However, an interesting feature of induced resistance that is common to both SAR and ISR, as well as to other types of induced resistance, such as that induced by BABA, is the phenomenon of priming. Here, plant defences are not directly activated by the inducing agent, but instead are potentiated for enhanced expression upon subsequent attack (Conrath 2009; Beckers and Conrath 2007, Conrath et al. 2006). Priming is considered to be less costly to plants than direct induction of resistance, since defences are only activated when required (Van Hulten et al. 2006; Walters et al. 2009). Indeed, priming is considered to represent a sensible approach to using induced resistance in crop protection (Conrath 2009).

Another feature of induced resistance is the fact that it does not provide complete disease control, but rather, reduces lesion size and severity (Kuć 1982). Greatest levels of disease control tend to be achieved under glasshouse conditions, with lower levels of control obtained on crops in the field (Vallad and Goodman 2004; Walters and Fountaine 2009). Since induced resistance is a host response, it will be influenced by host genotype and environment, and indeed, plant variety and crop nutrition are known to affect the expression of induced resistance (Walters and Fountaine 2009). However, under field conditions, another factor which might influence the expression of induced resistance is the likelihood that plants are already induced (Walters 2009). After all, plants in the field will interact with a variety of organisms, including microbial pathogens, beneficial microbes (e.g. mycorrhizal fungi), and herbivorous insects, all of which can induce resistance in plants (Walters 2009). Interestingly, elevated levels of defence gene expression in field-grown plants, prior to elicitor application, have been reported (Pasquer et al. 2005; Herman et al. 2007). It has been suggested that plants in the field already expressing induced resistance, activated via interactions with other organisms, might have a reduced ability to increase resistance further, for example following application of an elicitor (Walters 2009). If this is correct, it could have important, negative implications for use of induced resistance to control crop diseases. Here, we examine the possibility that prior infection with a pathogen can affect the ability of the plant to respond to application of resistance elicitors. The system used was barley (*Hordeum vulgare* L.) and the fungal pathogen *Rhynchosporium secalis,* the cause of leaf scald. We also used a combination of resistance elicitors (ASM, β-aminobutyric acid, and *cis*-jasmone), which has been shown previously to provide significantly greater control of foliar pathogens on barley than the elicitors used singly (Walters et al. 2010). The results show that prior infection with *R. secalis* can reduce the ability of the plant to express induced resistance further.

Materials and methods

Plant growth and pathogen inoculation

Seeds of barley (*Hordeum vulgare* L. cv Cellar) were sown in pots in Fisons Levington compost and grown

in a walk-in growth room at 18°C with a 16 h photoperiod (190 μmol m^{-2} s^{-1} provided by 400 W mercury vapour lamps). Plants were used for experiments when the third leaf was fully formed and the fourth leaf emerging. To create the 'already induced' state, leaves one and two were inoculated with the leaf scald pathogen, *Rhynchosporium secalis,* by spraying with a suspension of spores (1×10^5 spores/ml) in distilled water containing 0.01% Tween 20. Leaves three and four were protected from the inoculation spray by shielding with a plastic sheet. Inoculated plants were then covered with plastic bags for 48 h (the first 24 h in the dark) and kept at 16°C to provide the conditions necessary for spore germination and early fungal development. Thereafter, the temperature of the growth room was increased to 18°C for the remainder of the experiment. Leaves one and two of control plants were not inoculated. One week after inoculation of leaves one and two (at this stage, no disease symptoms were visible on these leaves), leaves three and four were sprayed to run-off with the elicitor combination (ASM + BABA + *cis*-jasmone; see below) using a hand-held sprayer. Leaves three and four were then inoculated with *R. secalis* 2 days later. Infection intensity was assessed 21 days after inoculation by visually determining the % leaf area exhibiting symptoms. At this stage, plants had 7–8 leaves; all leaves younger than leaf 2 (i.e. leaves 3–8) were used for assessment of infection. For gene expression and enzyme analyses, leaves three and four were harvested 1 day after inoculation and frozen at −80°C.

The elicitors used in these experiments were ASM (1 mM), BABA (1 mM), and CJ (0.625 g/l). ASM (Bion®) was a gift from Syngenta, Basel, Switzerland; BABA was purchased from Sigma, Poole, Dorset, UK; CJ was purchased from Sigma Aldrich, Dorset, UK.

Gene expression

Total RNA was extracted from barley leaves three and four using a RNeasy™ kit (Qiagen, West Sussex, UK) and RNA yield determined using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). In order to remove any remaining trace of DNA likely to interfere with measurements, samples were treated with desoxyribonuclease enzymes using the DNA-*free*™ kit from Applied Biosystems (California, USA). The final quantity and quality of the RNA was tested using a RNA 6000 Nano Chip kit (Agilent Technologies, Santa Clara, CA, USA).

Primer sequences for *PR1-b, LOX2* and the cyclophilin gene (internal control) are listed below:

PR1-b (forward): CTACGACTACGGCTCCAA CAC
PR1-b (reverse): GCATCACGGTTAGTA TGGTTTCTG
[Amplicon length: 190 base pairs]
LOX2 (forward): CGGCAGACTCCCTCAT CACTAAAG
LOX2 (reverse): GGCAGCAACAGGTCGTGG TAG
[Amplicon length: 121 base pairs]
Cyclophilin (forward): CCTGTCGTGTCG TCGGTCTAAA
Cyclophilin (reverse): ACGCAGATCCAG CAGCCTAAAG
[Amplicon length: 122 base pairs]

All sequences were purchased from Eurofins MWG Operon (Ebersburg, Germany) and all primers were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, California, USA).

Following RNA extraction, cDNA was generated using a SuperScript™ first-strand cDNA synthesis kit (Invitrogen, USA). Quantitative real-time PCR (qRT-PCR) was then performed with a MX3000P system (Stratagene, CA, USA) using a Brilliant 11SYBR Green QPCR master mix with ROX (Agilent Technologies, Santa Clara, CA, USA). In order to construct standard curves for the genes, six data points were used with a 5-fold dilution series (1:10 to 1:31,250). A 25 μl reaction for PCR amplification contained 12.5 μl of SYBR Green master mix (see above), 0.75 μl forward primer, 0.75 μl reverse primer, 6 μl sterile distilled water, and 5 μl cDNA. All PCR reactions were performed in duplicate. The cycling conditions were as follows: pre-incubation for 10 min at 95°C, followed by 40 cycles, each consisting of 30 s denaturing at 95°C, 60 s annealing at 57°C, 30 s at 72°C for new strand synthesis. The standard curves were used to calculate the absolute quantity of the product in each sample, Relative expression values were then calculated by normalising against the cyclophilin gene as an internal control.

Enzyme assays

Leaves three and four were harvested and frozen immediately in liquid nitrogen for subsequent enzyme analysis. The activities of the defence-related enzymes cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) and peroxidase (POX; EC 1.11.1.7) were determined as described by Boyle and Walters (2006), while β-1,3-glucanase (EC 3.2.1.6) was determined as described by Heil and Ploss (2006). The enzyme assays were scaled down in order to allow them to be carried out using 96-well microtitre plates.

Statistical analysis

All experiments were repeated with similar results and data were subjected to ANOVA using the GenStat Release 11.1 statistical program. % leaf area infected values from glasshouse experiments were log-transformed prior to analysis. Comparison of treatment means was performed using Fisher's protected least significant difference (LSD) Test. For the infection assessment experiments, 10 replicates were used, while three replicates were used in the gene expression and enzyme activity experiments.

Results

Prior inoculation of barley with *R. secalis* reduces the effectiveness of elicitors to control further infection

To test whether existing infection with a pathogen alters the ability of the plant to express induced resistance further, first and second leaves of barley were inoculated with *R. secalis* and left for 1 week before the elicitor combination was applied to leaves three and four. Two days later, leaves three and four were inoculated with *R. secalis.* The treatments in this experiment were: [1] leaves one and two not inoculated, no elicitor applied to leaves three and four, leaves three and four inoculated (NI+NE+I); [2] leaves one and two not inoculated, elicitor applied to leaves three and four, leaves three and four inoculated (NI+E+I); [3] leaves one and two inoculated, elicitor applied to leaves three and four, leaves three and four inoculated (I+E+I); and [4] leaves one and two inoculated, elicitor not applied to leaves three and four, leaves three and four inoculated (I+NE+I). In plants where the first two leaves were not inoculated, treatment of leaves three and four with elicitor led to a 67% reduction in *R. secalis* infection of leaves three and four (NI+E+I), compared to plants receiving no elicitor treatment (NI+NE+I) (Fig. 1). In contrast, if leaves one and two were inoculated prior to treatment of leaves three and four with elicitor, there was no reduction in *R. secalis* infection (treatment I+E+I) (Fig. 1). These data indicate that if barley plants are already infected, they do not respond effectively to application of elicitors and disease control is compromised. Interestingly, these data also showed that although prior inoculation with *R. secalis* reduced further infection by the same pathogen (I+NE+I), this reduction was not significant (Fig. 1).

PR1-b is up-regulated and *LOX2* down-regulated by prior inoculation and elicitor treatment

The mechanisms underlying the effects of prior inoculation on the expression of induced resistance observed in Fig. 1 were examined by following the expression of two defence-related genes, *PR1-b,* which is used as a marker gene for SAR, and *LOX2,* which is involved in JA biosynthesis.

PR1-b was not expressed in plants following a number of treatments, notably plants which received no treatment (NI+NE+NI), and plants where leaves

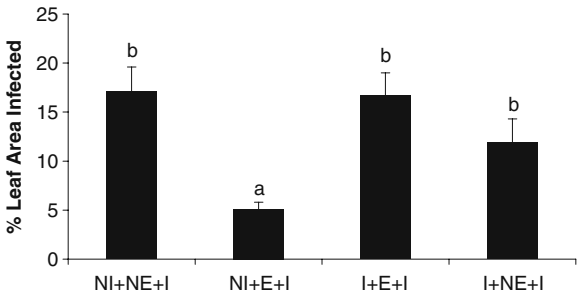

Fig. 1 Effects of prior inoculation with *R. secalis* on the ability of barley to respond to subsequent elicitor application. *R. secalis* infection is shown for leaves 3–8. Treatments were: [1] leaves one and two not inoculated, leaves three and four not treated with elicitor but inoculated (NI+NE+I), [2] leaves one and two not inoculated, leaves three and four treated with elicitor and inoculated (NI+E+I), [3] leaves one and two inoculated, leaves three and four treated with elicitor and inoculated (I+E+I), [4] leaves one and two inoculated, leaves three and four not treated with elicitor but inoculated (I+NE+I). Bars with different letters are significantly different at $P<0.05$ (Fisher's LSD)

one and two had been inoculated with *R. secalis* (I+NE+NI). These treatments do not appear in Figs. 2 and 3. Inoculation of leaves three and four (NI+NE+I) led to expression of *PR1-b* in those leaves, as did application of the elicitor combination to leaves three and four (NI+E+NI) (Fig. 2a, b). If the latter leaves were subsequently inoculated (NI+E+I), there was a further increase in *PR1-b* expression, indicating that the elicitor combination primed the plant for enhanced *PR1-b* expression (Fig. 2a, b). However, when leaves one and two were inoculated prior to treatment of leaves three and four with elicitor (treatments I+E+NI and I+E+I), expression of *PR1-b* was reduced (Fig. 2a, b). These data suggest that prior infection reduces the ability of the plant to activate SAR following elicitor application. Inoculation of leaves one and two, followed by inoculation of leaves three and four (I+NE+I) led to weak expression of *PR1b* in leaves three and four (Fig. 2a, b).

In contrast, all treatments resulted in a down-regulation of *LOX2* (Fig. 3). For example, inoculation of leaves three and four (NI+NE+I) led to a seven-fold reduction in *LOX2* expression, while *LOX2* expression was reduced between three- and five-fold following elicitor treatment (NI+E+NI) (Fig. 3a, b). Taken together, these data suggest that *R. secalis* infection of leaves three and four up-regulates *PR1-b* in those leaves, but down-regulates *LOX2* in these leaves. Similarly, treatment with the elicitor combination up-regulates *PR1-b,* but results in a massive down-regulation of *LOX2.*

Expression of defence-related enzymes is compromised by prior inoculation of elicitor-treated plants

In order to determine whether up-regulation of *PR1-b* was accompanied by increased defences, the activities of three defence-related enzymes, cinnamyl alcohol dehydrogenase (CAD), peroxidase (POX) and β-1,3-glucanase were examined in response to the various treatments. Additional treatments shown in Figs. 4, 5

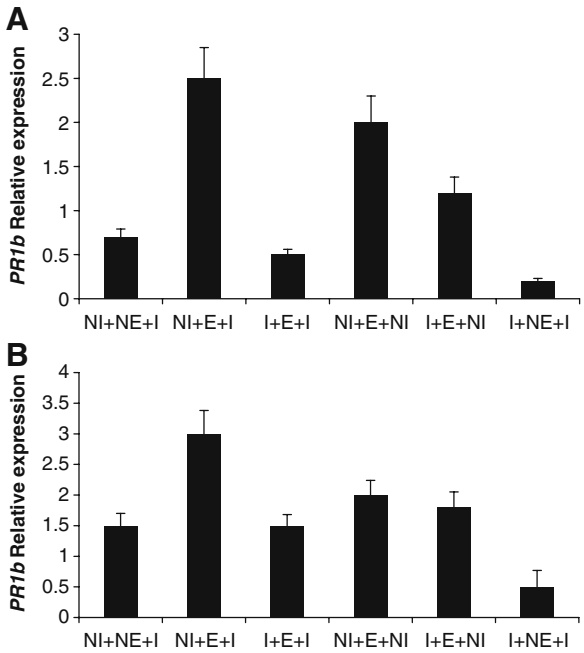

Fig. 2 Expression of *PR1-b* in leaves three (**a**) and four (**b**) following prior inoculation of leaves one and two. Treatments were: [1] leaves one and two not inoculated, leaves three and four not treated with elicitor but inoculated (NI+NE+I), [2] leaves one and two not inoculated, leaves three and four treated with elicitor and inoculated (NI+E+I), [3] leaves one and two inoculated, leaves three and four treated with elicitor and inoculated (I+E+I), [4] leaves one and two not inoculated, leaves three and four treated with elicitor but not inoculated (NI+E+NI), [5] leaves one and two inoculated, leaves three and four treated with elicitor but not inoculated (I+E+NI), and [6] leaves one and two inoculated, elicitor not applied to leaves three and four, but these leaves inoculated (I+NE+I). Bars with different letters are significantly different at $P<0.05$ (Fisher's LSD)

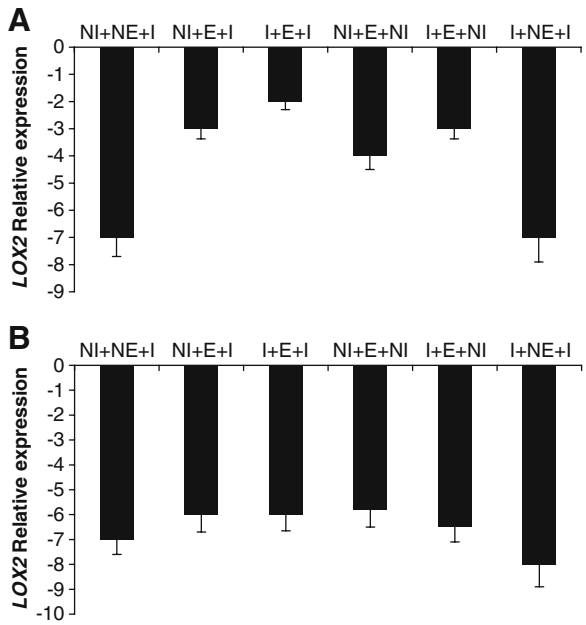

Fig. 3 Expression of *LOX2* in leaves three (**a**) and four (**b**) following prior inoculation of leaves one and two. Treatments as in the legend to Fig. 2. Bars with different letters are significantly different at $P<0.05$ (Fisher's LSD)

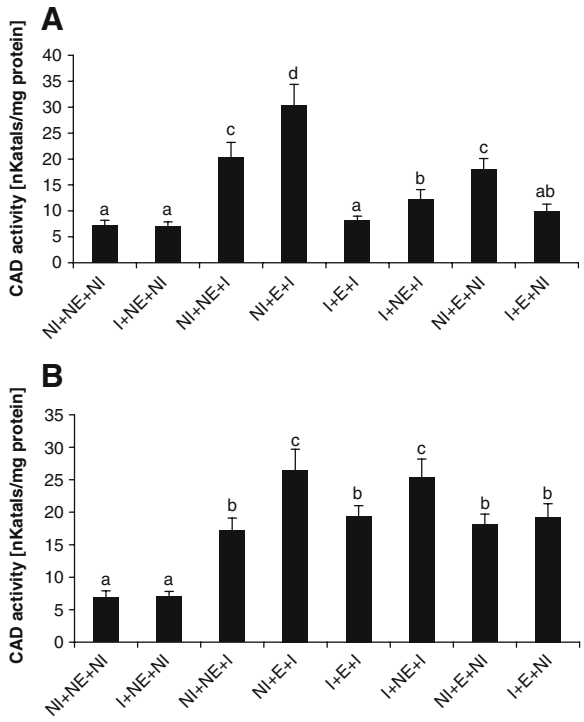

Fig. 4 CAD activity in leaves three (**a**) and four (**b**) following prior inoculation of leaves one and two. Treatments were: [1] leaves one and two not inoculated, leaves three and four not treated with elicitor nor inoculated (NI+NE+NI), [2] leaves one and two inoculated, leaves three and four not treated with elicitor nor inoculated (I+NE+NI), [3] leaves one and two not inoculated, leaves three and four not treated with elicitor but inoculated (NI+NE+I), [4] leaves one and two not inoculated, leaves three and four treated with elicitor and inoculated (NI+E+I), [5] leaves one and two inoculated, leaves three and four treated with elicitor and inoculated (I+E+I), [6] leaves one and two inoculated, leaves three and four not treated with elicitor but inoculated (I+NE+I), [7] leaves one and two not inoculated, leaves three and four treated with elicitor but not inoculated (NI+E+NI), [8] leaves one and two inoculated and treated with elicitor, but leaves three and four not inoculated (I+E+NI). Bars with different letters are significantly different at $P<0.05$ (Fisher's LSD)

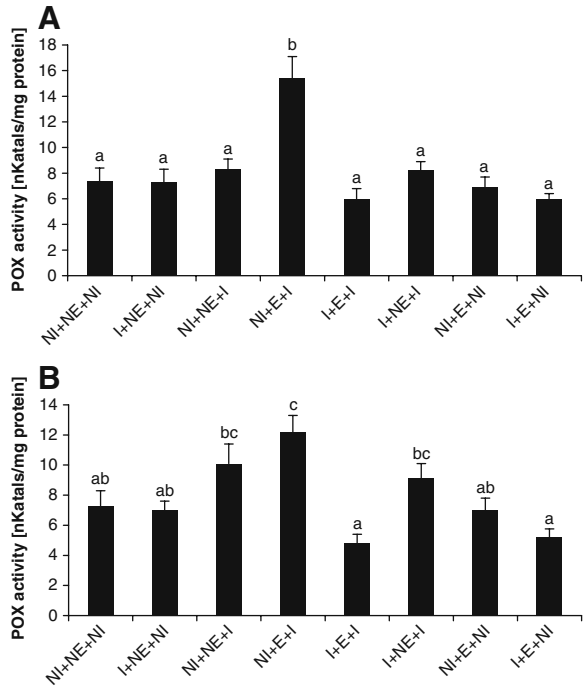

Fig. 5 POX activity in leaves three (**a**) and four (**b**) following prior inoculation of leaves one and two. Treatments as in the legend to Fig. 4. Bars with different letters are significantly different at $P<0.05$ (Fisher's LSD)

and 6, but not shown in Figs. 2 and 3, are untreated plants (NI+NE+NI), and plants with leaves one and two inoculated with *R. secalis* (I+NE+NI).

Application of the elicitor combination to leaves three and four (NI+E+NI) increased CAD activity in these leaves compared to untreated plants (NI+NE+NI) (Fig. 4a, b). When these leaves were subsequently inoculated with *R. secalis* (NI+E+I), there was a further, significant, increase in CAD activity in both leaves, suggesting that the elicitor combination primed the plant for enhanced CAD activity (Fig. 4a, b). Inoculation of leaves one and two before elicitor treatment of leaves three and four (I+E+NI and I+E+I) led to a significant reduction in CAD activity in the third and fourth leaves (Fig. 4a, b). With POX, treatment of leaves three and four with elicitors (NI+E+NI) did not alter enzyme activity compared to untreated plants (NI+NE+NI) (Fig. 5c, d). In contrast, if elicitor treated leaves were then inoculated (NI+E+I), POX activity was increased significantly. However, this increase in POX activity did not occur if leaves one and two had been previously inoculated (I+E+NI and I+E+I) (Fig. 5c, d). The trend for glucanase activity was similar to that for CAD, in that treatment of leaves three and four (NI+E+NI) increased enzyme activity in these leaves compared to the untreated control (NI+NE+NI) (Fig. 6e, f). However, there was no further increase in glucanase activity if elicitor treated plants were then inoculated with *R. secalis* (NI+E+I), although prior inoculation of leaves one and two (I+E+NI and I+E+I) did result in a significant reduction in enzyme activity in leaves three and four (Fig. 6e, f).

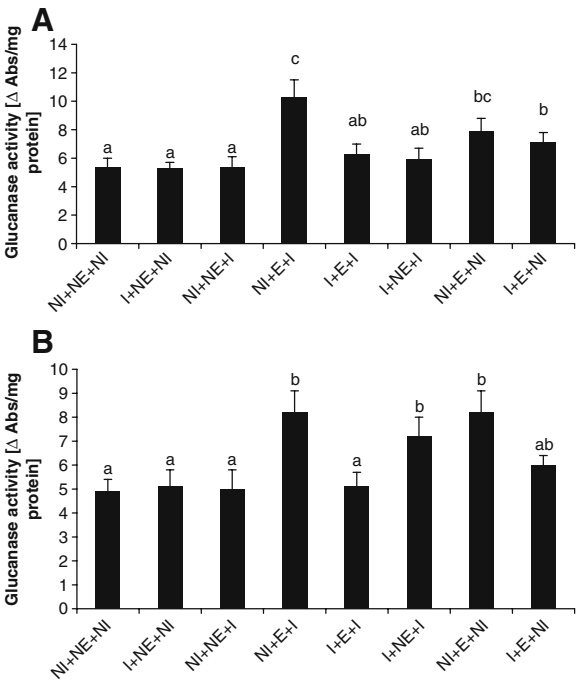

Fig. 6 Glucanase activity in leaves three (**a**) and four (**b**) following prior inoculation of leaves one and two. Treatments as in the legend to Fig. 4. Bars with different letters are significantly different at $P<0.05$ (Fisher's LSD)

The results for CAD, POX and glucanase suggest that prior inoculation of leaves one and two with *R. secalis* compromises the ability of the plant to increase enzyme activities in response to subsequent elicitor treatment.

Discussion

Under controlled conditions, induced resistance can provide substantial reductions in pathogen infection (Walters and Reignault 2007). Under field conditions, elicitors can also provide effective disease control. Thus, BABA has been shown to provide good control of downy mildew in grapevines (Reuveni et al. 2001), and late blight in potato and tomato (Cohen 2002, Liljeroth et al. 2010). However, for some crops under field conditions, disease control is often less effective (Vallad and Goodman 2004; Walters et al. 2005). Although the efficacy of induced resistance can be influenced greatly by plant variety and environmental and agronomic conditions, it has been suggested that induced resistance might be less effective under field conditions because plants are already induced (Walters 2009). It would be extremely difficult to examine this suggestion under field conditions, and so it was decided to conduct experiments under controlled conditions, using prior inoculation with a foliar pathogen to simulate plants in the field environment.

The elicitor combination contained ASM, which is widely reported to activate SAR and to lead to the coordinate expression of a specific set of genes encoding pathogenesis-related (PR) proteins (Ryals et al. 1996). The situation regarding BABA, which was also a component of the elicitor combination, is more complex, since it appears to induce resistance via SA-dependent, SA-independent, and ABA-dependent mechanisms, with the relative importance of the different mechanisms depending upon the nature of the attacking pathogen (Ton et al. 2005). CJ is structurally related to JA and methyl-JA, although it up-regulates a unique set of genes compared to methyl-JA (Birkett et al. 2000; Pickett et al. 2007). When applied to barley, the elicitor treatment led to an up-regulation of *PR1-b,* suggesting that it activates SAR in barley. Moreover, up-regulation of *PR1-b* was accompanied by an even more pronounced down-regulation of *LOX2*. The *LOX2* gene, which is involved in the octadecanoid pathway, is auto-regulated by JA, thereby controlling a feed-forward loop in JA biosynthesis (Bell et al. 1995). Indeed, suppression of *LOX2* in transgenic *Arabidopsis* was shown to block JA biosynthesis during pathogen infection (Spoel et al. 2003). The results presented in this paper suggest that in barley, the elicitor combination activates SA-mediated SAR, but suppresses JA signalling. The suppression of JA signalling by activation of SA-mediated SAR has been well documented (Bostock 2005; Pieterse and Van Loon 2007). This is likely to prioritise SA-dependent resistance to biotrophic pathogens over JA-dependent defence that tends to be more effective against necrotrophic pathogens and herbivorous insects (Bostock 2005). For example, ASM induced resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato,* but increased herbivory by larvae of the corn earworm, *Helicoverpa zea* (Stout et al. 1999). Less is known about pathogens, although infection of *Arabidopsis thaliana* by the biotrophic pathogen *P. syringae* DC3000, was found to increase susceptibility to the necrotrophic pathogen *A. brassicicola,* brought about via SA suppression of JA-

responsive gene expression (Spoel et al. 2007). The reduction in *R. secalis* infection in elicitor-treated plants probably reflects the hemi-biotrophic nature of the pathogen, which has an initial biotrophic phase, followed by a necrotrophic phase (Walters et al. 2008). Interestingly, *R. secalis* infection also resulted in down-regulation of *LOX2* in barley, raising the possibility that infected plants might be compromised in their ability to protect themselves against necrotrophic pathogens and herbivorous insects. Clearly, the consequences of the pathogen- and elicitor-induced down-regulation of *LOX2* for interactions of barley with necrotrophic pathogens and insect pests warrant investigation.

Compared to plants where leaves three and four were inoculated with *R. secalis,* prior treatment with the elicitor combination led to a significant reduction in infection of these leaves. Interestingly, if leaves one and two were inoculated before elicitor application to leaves three and four, this protective effect disappeared. However, prior inoculation of leaves one and two, followed by later inoculation of third and fourth leaves, led to a 36% reduction in infection, although this effect was not significant. This suggests that prior inoculation with *R. secalis* did not induce resistance. Indeed, an 'already induced' state might be expected to be reflected in enhanced defensive activity compared to the non-induced state. However, activities of the defence-related enzymes CAD, POX and β-1,3-glucanase in plants with leaves one and two inoculated with *R. secalis* were not elevated above those observed in untreated plants, and neither was *PR1-b* expressed in these plants. This might reflect the fact that the first two leaves of barley were used to create the 'already induced' state, because inoculation of leaves three and four did lead to elevated CAD activity and to an up-regulation of *PR1-b.* Moreover, under field conditions, where plants experience numerous, simultaneous interactions with other organisms, expression of defence genes and elevated activities of defence-related enzymes, are detected, even in the absence of exogenous treatments (Pasquer et al. 2005; Herman et al. 2007).

Treatment of barley with the elicitor combination resulted in disease control, up-regulation of *PR1-b,* and increased activities of defence-related enzymes. Further, subsequent inoculation of elicitor-treated plants led to further increases in expression of *PR1-b,* and activities of CAD and POX. This suggests that the elicitor combination primes barley for enhanced expression of these defence responses. That the elicitor combination leads to priming in barley should be no surprise, since both ASM and BABA are known to prime various plants for enhanced defence responses (e.g. Ton and Mauch-Mani 2004; Van Hulten et al. 2006).

As indicated earlier, it has been suggested that if plants in the field are already induced, they might not be able to respond effectively to application of elicitors (Walters 2009). To date, this has not been tested. In this paper, we used prior inoculation of the first two leaves of barley in an attempt to create an 'already induced' state. The resulting data show quite clearly, that the enhanced levels of disease control, *PR1-b* expression, and activities of the defence-related enzymes CAD, POX and glucanase in leaves three and four of elicitor-treated plants, are reduced substantially and significantly, if the first two leaves are already infected with *R. secalis.* However, the failure of prior inoculation of the first two leaves to provide protection to leaves three and four, together with the lack of any defence activation in plants with the lower two leaves infected, suggests that these plants might not be induced. Rather, the data suggest that prior inoculation of leaves one and two with *R. secalis* suppresses defence to subsequent infection in leaves three and four. In this case, prior infection would also compromise the effect of the elicitor combination by making it more difficult for the SA-mediated defences to be activated. Pathogen suppression of host defences is well known, such as the differential suppression of defence genes demonstrated in interactions between potato and different isolates of the Oomycete pathogen, *Phytophthora infestans* (Wang et al. 2006, 2008). Indeed, pathogens are capable of hijacking the plant's defence signalling network in order to manipulate the host immune response (Grant and Jones 2009). Thus, SA has been widely reported to antagonise JA-dependent plant defences, while JA has also been shown to antagonise the SA pathway (Diezel et al. 2009; Koorneef and Pieterse 2008). The plant hormone abscisic acid (ABA) is now also known to be involved in cross-talk in plant defence. *P. syringae* was shown to alter host ABA levels, thereby suppressing SA biosynthesis and action, leading to enhanced susceptibility (De Torres-Zabala et al. 2007, 2009). In view of the data presented in this paper, there would be merit in

determining whether *R. secalis* is indeed capable of suppressing SA-mediated defences in barley.

Irrespective of the mechanisms underlying these responses, these data show, for the first time, that barley plants that are already infected with *R. secalis* are compromised in their ability to respond effectively to elicitor application. Whether these results, obtained using young barley plants under growth room conditions, are transferable to a field-grown barley crop, is not known. Different responses might be obtained with different barley varieties, with different pathogens, or with different elicitors. It would seem prudent to determine whether these results hold true for pathogens other than *R. secalis* and for elicitors other than those used in this study.

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