Changes in dihydroxymethoxybenzoxazinone glycoside content in wheat plants infected by three plant pathogenic fungi

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(*Accepted for publication March 1995*)

Hydroxamic acids (Hx), present among members of the family Gramineae, defend the plant against several insect species and some bacteria. Their role in defence against fungal pathogens is still unclear. We tested the hypothesis that the mode of infection by the fungus influences the release from DIMBOA-glucoside, the main Hx in wheat, of the more active aglycone DIMBOA. Infection by the pathogenic necrotroph *Septoria tritici* resulted in significant hydrolysis of DIMBOA-glucoside in seedlings of two wheat cultivars differing in Hx concentrations. Infection by *Drechslera teres*, a necrotroph non-compatible with wheat but pathogenic on barley, caused only a slight reduction of glucoside concentration. The content of aglycone did not increase concurrently in any of these experiments. No change in glucoside content was evident following infection by the obligate parasite *Puccinia recondita*. *In vitro*, *S. tritici* was able to increase by 39% the rate at which DIMBOA disappeared from aqueous solutions. Free DIMBOA was not detected in roots of plants infected with *S. tritici*, but there was a tendency for glucoside levels to be higher than in roots of control plants.

INTRODUCTION

Plants respond to attack by fungal pathogens in a number of different ways. Among the biochemical responses, the most rapid are increases in enzymatic activity including peroxidases [*16*, *19*] phenylalanine ammonia lyase (PAL) [*31*, and refs. therein]. Other reactions include the accumulation of phytoalexins, and of pathogenesis-related proteins [*31*]. Recently, salicylic acid concentration has been shown to increase upon fungal infection and it is suggested to be involved in eliciting systemic defence responses [*24*, *35*].

A group of secondary plant substances, the hydroxamic acids (Hx), is present as glucosides in the tissues of various wild and cultivated gramineae [*25*]. Cell damage leads to enzymatic hydrolysis of the glucosides yielding free aglycones [*15*] which are deleterious to a range of different organisms including insects [*9*, *2*, *17*] and bacteria [*30*]. Additionally, Hx exuded from rye roots substantially suppressed growth of wild oats (*Avena fatua*) [*27*], indicating strong allelopathic effects.

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Abbreviations used in text: DIMBOA, 2,4-dihydroxy-7methoxy-1,4-benzoxazin-3-one; Hx, hydroxamic acids; PAL phenylalanine ammonia lyase; YMA, yeast malt agar; PDA, potato dextrose agar; PVP, polyvinyl pyrrolidone; PMSF, phenylmethylsulfonylfluoride; GLC, DIMBOA glucoside.

0885-5765/95/090201+12 $12.00/0

Efforts to relate Hx content in plants (mainly maize and wheat) to the level of fungal resistance have given ambiguous results. Early work, reporting positive correlations between resistance and concentration of Hx [*8*, *20*], was later refuted [*12*, *18*] when the relationships were looked upon in more detail and a wider genotypic range of the host plant was used. Studies showing lack of correlation includes that on resistance in wheat against *Helminthosporum sativum* [*10*] and in maize against *Colletotrichum graminicola* [*22*], while those showing negative correlations between Hx and fungal incidence includes that on *Sphacelotheca reilina* in maize [*36*]. Furthermore, although some species of fungi show sensitivity towards Hx *in vitro* (*Fusarium nivale* [*34*], *Gaeumannomyces graminis*—F. J. Pérez, pers. comm.) such reactions do not reflect reactions *in vivo* and may just give spurious correlations.

Feeding by insects may induce changes in the context of Hx. Thus, concentrations of DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) in maize and wheat extracts increased following damage by the chewing insect *Sesamia nonagrioides* [*13*] and sucking insect *Metopolophium dirhodum* [*26*]. Recently, Bücker and Grambow [*4*] reported similar changes in another Hx derivative, N-O-Me-DIMBOA (2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one), upon infection of wheat by wheat stem rust (*Puccinia graminis*). While levels of Hx declined during plant development, the concentration of N-O-Me-DIMBOA increased in a partially resistant cultivar up to 5 days post-inoculation and dropped thereafter. No such induction was found in highly resistant or susceptible cultivars.

In this work we tested the hypothesis that free DIMBOA aglucone released in the plant tissues upon fungal invasion is dependent on the mode of penetration by the fungus. We measured simultaneously glucoside and aglucone levels in healthy plants and in plants inoculated with three species of fungi exhibiting different invasive behaviour: the pathogenic necrotroph *Septoria tritici*, the non-pathogenic necrotroph *Drechslera teres* and the pathogenic obligate parasite *Puccinia recondita*. We hypothesized that the two necrotrophs, through their cell-damaging mode of penetration, would provoke glucoside hydrolysis with aglucone production, while the obligate parasite, showing a specific biotrophic relationship with its host, would not.

MATERIALS AND METHODS

Plant and fungal cultures

Seeds of wheat (*Triticum aestivum* L.) cv. Millaleu (low to medium Hx levels [*5*]), was obtained from INIA/Chillán, S. Chile. The soil used for growing all plants was Tierra de Hoja (ANASAC). Ambient conditions in the growth room were 16:8 h (light:dark), 24 °C with a 4° range and 85 % relative humidity *Septoria tritici* Rob. in Desm. (obtained from INIA/Chillán) was cultivated on yeast-malt Agar (YMA; 9 g each of yeast extract, malt extract and dextrose—all from Difco—plus 15 g of agar l^{-1} of medium) or liquid YM-solution (as above but without agar). *Drechslera teres* Shoem., which together with *Puccinia recondita* Rob. ex Desm. was isolated from infected fields outside Santiago, was grown on potato dextrose agar (PDA—Difco). *P. recondita* (race not determined) was maintained on the susceptible wheat cv. Millaleu. The identity of all pathogens was confirmed after reinoculation on susceptible plants.

Test of infection success

The post-inoculation times (maximum 11 days) were not long enough to allow visual symptoms to appear on the plants. Therefore, to ensure that all pathogens actually elicited defence responses in the wheat plants, total peroxidase activity was measured in infected and uninfected plants. Inoculum of *S. tritici* or *D. teres* was prepared by mixing and heating until dissolution 1 g of gelatin powder in 200 ml deionized water. Following addition of another 200 ml water, the solution was divided equally between two beakers. Agar cultures from Petri plates (inoculated or uninoculated) were added to each beaker and homogenized with an Ultra-Turrax at 800 rpm for 2 min before filtering through gauze into a hand sprayer. The amount of inoculum was sufficient for two consecutive sprayings of four plants in pots every second day. After inoculation each pot was covered with a plastic bag and sealed. Infections with *P. recondita* were made by gently pulling each leaf between the fingertips that had previously been moistened and smeared with uredospores. Control plants were treated in essentially the same way. The infection process was facilitated by spraying with water and covering the pot with a thin plastic bag.

Analysis of peroxidase activity was performed as follows. Seventy-two hours after initial inoculation, 5 g of biomass were harvested from each pot and homogenized with 5 ml water +2 ml of buffer pH 7·2 containing 0·25 M saccharose, 5 mM $MgCl_2$, 50 mM Tris-HCl, 1% PVP (polyvinyl pyrrolidine, SIGMA), 0·1 mM PMSF (phenylmethylsulfonylfluoride, SIGMA) using an Ultra-Turrax at 1600 rpm. After filtration through gauze, samples were centrifuged at 1000 *g* for 5 min (Beckman J2-21), the supernatants removed and frozen until required for analysis. The reaction was started by mixing 5–10 µl of sample, depending on the amount of activity, 20 µl 92·5 mM *o*-phenylenediamine, (SIGMA), 12 µl H_2O_2 (10%) and 50 mM Tris-phosphate buffer (pH 6·8) to a total of 1000 µl. Absorbance at 460 nm was measured with a Shimadzu UV-240, 1, 2, 3 and 4 min after mixing and activity was determined from the slope of absorbance against time.

Inoculation experiments

Wheat seeds were sown in plastic trays containing wells (3 × 3 × 6 cm) at 3 seeds per well. Five days later, on the day of inoculation (day 0), the plants were thinned to one per well to obtain uniformity and six control plants from each cultivar were harvested for simultaneous analysis of DIMBOA glucoside (GLC) and aglycone. Inoculum was prepared as described, sprayed on the plants until run-off on two consecutive days. Infected and uninfected plants were placed in separate compartments under a plastic tent which was sealed to keep humidity at maximum. Six plants per cultivar per treatment were withdrawn for analysis on days 2, 4, 5, 7 and 9 following inoculation. Conditions for light and temperature were as described earlier. An identical set of experiments were also made using the wheat cv. Lautaro (medium to high Hx content), to test the hypothesis in plants with two different Hx levels.

Sample preparation

Before extraction, plant height was measured and the fresh weight estimated from a linear regression of height *vs.* fresh weight ($R^2 = 0{\cdot}96$). The plants were cut just above

the coleoptile and immediately macerated using mortar and pestle with approx. 300 mg sea sand (Merck) in 1·0 ml 0·1 M glycine-HCl buffer pH 2. Complete maceration took less than 8 s. Control experiments with known amounts of both GLC and DIMBOA added to wheat, barley and oat extracts confirmed that neither compound decomposed during the extraction procedure. For the sake of handling and rapid maceration, 100 mm segments (measured from the tip of the leaf) were sampled from leaves longer than 10 cm, beginning 5 days post-inoculation. Extracts were centrifuged at 12000 rpm for 10 min and analyzed subsequently or stored in the freezer until analysis, which was never more than 48 h after centrifugation. Preliminary work showed both GLC and DIMBOA to be stable in this buffer over a range of temperatures (up to 26 °C) and storage times (up to 7 days).

Analysis of Hx in roots and leaves

Five germinated 2-day-old wheat seeds were placed 2 cm apart along the edge of a wet filter paper strip (4 × 25 cm) so that the coleoptiles extended above the long edge. Subsequently, the strip was folded along the long edges, rolled and inserted into a 15 × 1·5 cm glass tube filled with tap water. Each tube was wrapped with aluminum foil to keep out light. A total of eight such tubes (40 seeds) were prepared. On day 5 post-germination, half of the seedlings were inoculated with a suspension of *S. tritici* spores and the remaining half treated with a control solution. Three days later the first leaf and the root mass were cut from 10 plants of each treatment, measured (leaves) or weighed (roots), and macerated in buffer as described earlier. No symptoms of deficiency were observed during this short period of experimentation.

Chromatographic analysis

Samples were analyzed using isocratic elution with a mixture consisting of 30% methanol (OmniSolv, EMScience) and 70% of a mixture of 1000 ml deionized water with 0·5 ml of 85% *o*-H_3PO_4. Flow rate was 1·5 ml min^{-1} and detection made at 263 nm. Mixed standard solutions were prepared from purified GLC and DIMBOA extracted from maize as described [*21*, 29]. Slopes of response curves had typical R^2-values greater than 0·98. The column employed was a LiChrosphere 100 RP-18 (5 mm I.D.; Merck) and the equipment consisted of LC-9A pumps, SPD-6A UV detector and CR4A integrator (all Shimadzu).

Benzoxazinone decomposing activity of aqueous extracts of S. tritici *and* D. teres

Fungal mycelium from a 2- or 4-week-old plate culture of *D. teres* or *S. tritici*, respectively, was scraped off, weighed, mixed with 2 ml distilled water pH 5·3, and centrifuged at 3000 ***g*** for 5 min. The supernatant was filtered twice through double filter paper (Whatman No. 2) and the resulting extract was stored at 4 °C, for no longer than 2 days.

Comparative measurements of activity were made in two ways. First, from measurements of peak areas from HPLC traces obtained under the conditions described above: 500 µl of extract were mixed with pure GLC or DIMBOA and at regular intervals over 3 h, 50 µl was injected. Rates of disappearance were calculated from plots of log (peak area) against time. Second, from measurements of absorbance between 220 and 400 nm (31 °C, pH 6·2) on the following solutions: (a) DIMBOA-GLC + 1 ml 0·1 M phosphate buffer, pH 6·2; (b) DIMBOA + 1 ml buffer; (c)

DIMBOA + 950 µl buffer + 50 µl *S. tritici* extract and (d) DIMBOA + 950 µl buffer + 50 µl autoclaved *S. tritici* extract. GLC and DIMBOA were present at either 20 or 40 µg ml^{-1} in all solutions. Readings were taken every 90 min during the first 4·5 h and the last reading 24 h later. Rates of disappearance were calculated from plots of absorbance at 263 nm against time.

Statistical analysis

Data from peroxidase activity measurements were subjected to linear regression analysis and differences between slopes tested according to Zar [*38*]. All other data relating to Hx analyses were subjected to ANOVA including cultivar and treatment as independent variables.

RESULTS

Analyses of total peroxidase activity

Inoculating wheat plants with either pathogenic fungi such as *S. tritici* or *P. recondita* or a non-pathogenic fungus isolated from barley, *D. teres*, resulted in an increase in total peroxidase activity compared with uninfected control plants within 3 days of inoculation (Table 1). This showed that although no leaf damage was visible, the fungi had penetrated the leaf tissue and biochemical changes had taken place in the leaves. Successful infection by *S. tritici* and *P. recondita* became evident as symptoms appeared towards the end of each experiment.

TABLE 1

Total peroxidase activity in control and treated wheat plants 72 h after inoculation

Pathogen	Slope (Abs_{460} min^{-1})		t (d.f.)	*P*
	Infected	Control		
Septoria tritici	0·364	0·293	5·909 (36)	< 0·001
Drechslera teres	0·472	0·371	4·316 (33)	< 0·001
Puccinia recondita	0·536	0·375	5·385 (36)	< 0·001

Analyses of DIMBOA glycoside and aglycone

As the results from both wheat cultivars studied corresponded closely in all plant-pathogen combinations, only the results from cultivar Millaleu are given. This also applies to the interpretations in the Discussion.

Septoria tritici. The GLC content was significantly lower in plants infected with this pathogenic necrotroph than in control plants ($F = 14·22$, $P < 0·001$; Fig. 1). Overall, total GLC content decreased in both control and infected plants and by the seventh day after inoculation the levels were similar in both treatments. There were no significant differences in DIMBOA content between the two plant groups. DIMBOA concentrations also decreased rapidly in the plants and by the fourth day post-inoculation the levels were close to zero.

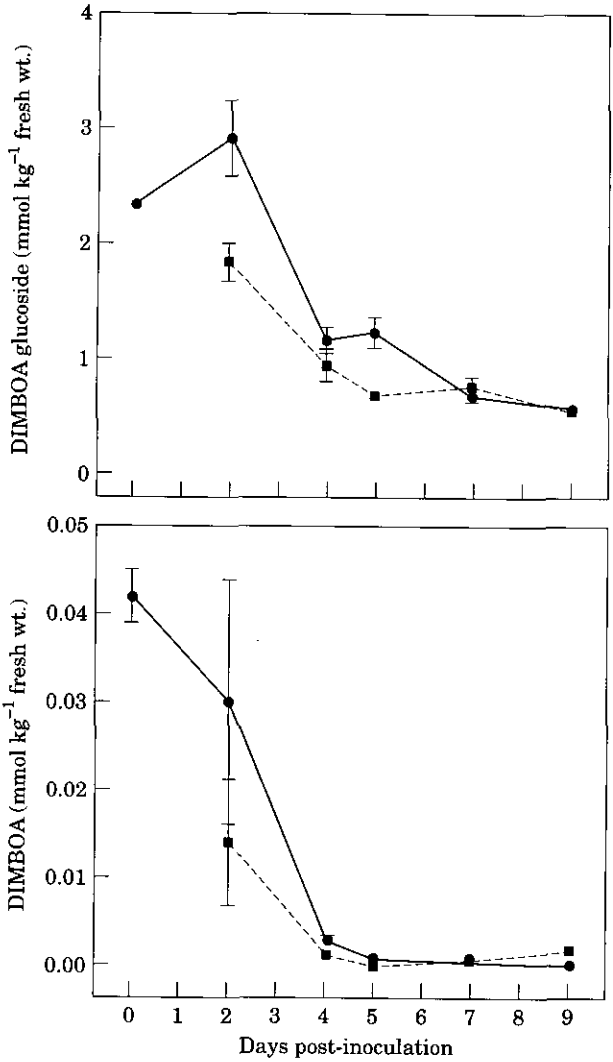

FIG. 1. Changes in content of DIMBOA glucoside and DIMBOA in wheat (*Triticum aestivum* cv. Millaleu) after inoculation with the wheat-compatible necrotroph *Septoria tritici*. Control plants (●), infected plants (■). Standard errors are indicated with bars except where included within the data point.

Drechslera teres. When plants were infected with this non-compatible necrotroph a slight but significant reduction of GLC ($P < 0{\cdot}05$) was observed (Fig. 2). The continuous decrease in total GLC-levels was again evident, as described above. The content of DIMBOA over the whole sampling period was also slightly reduced ($P < 0{\cdot}05$) in inoculated plants compared to the controls (Fig. 2), but due to large variation there were no significant differences between treatments on any individual date. As in the first experiment, DIMBOA content decreased rapidly in both plant groups.

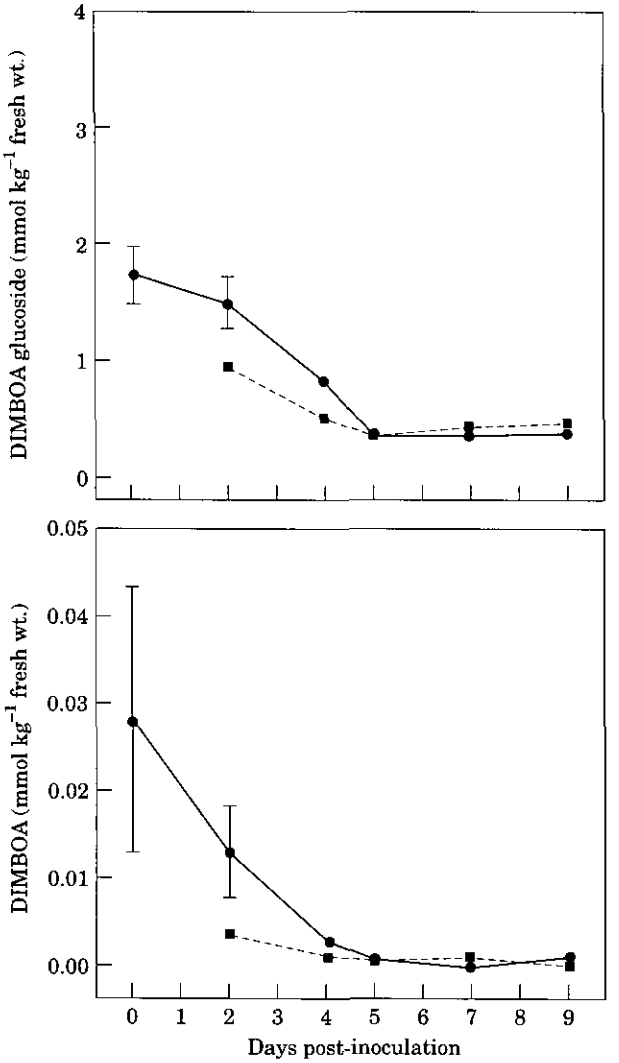

FIG. 2. Changes in content of DIMBOA glucoside and DIMBOA in wheat (*Triticum aestivum* cv. Millaleu) after inoculation with the wheat-incompatible necrotroph *Drechslera teres*. Control plants (●), infected plants (■). Standard errors are indicated with bars except where included within the data point.

Puccinia recondita. No effects were evident after inoculation by this obligate parasite. The general pattern of decreasing GLC content with plant development, as seen in the previous two experiments, was again obvious (Fig. 3). DIMBOA content of infected plants was slightly lower ($P < 0{\cdot}05$) than in control plants. This, however, was due to small differences on day 2 post-inoculation in the development between the two groups, as control seedlings emerged later (mean seedling sizes were 75 and 86 mm for control and infected groups, respectively).

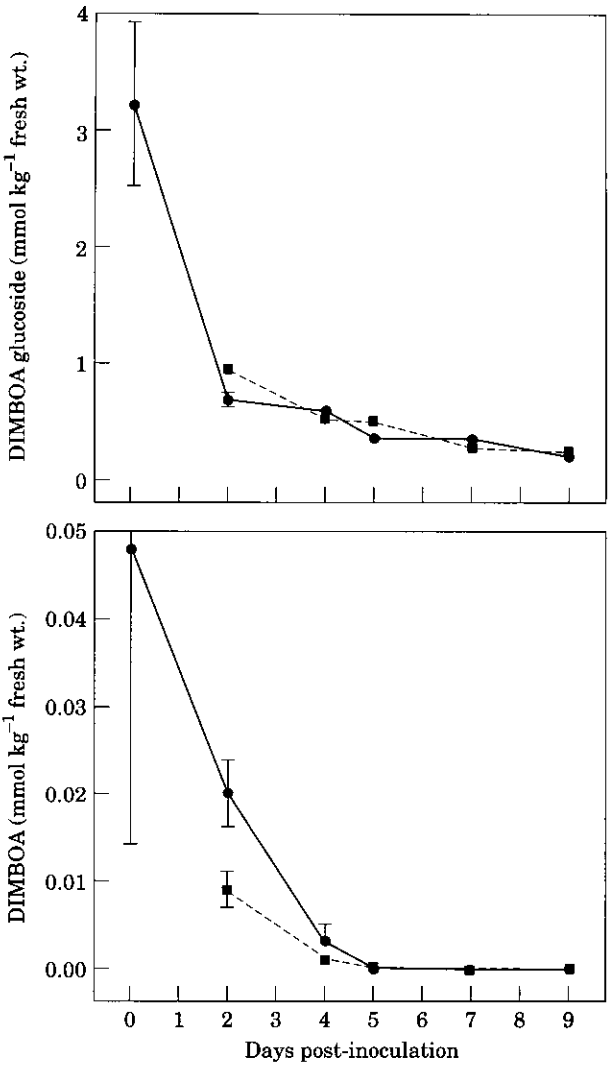

FIG. 3. Changes in content of DIMBOA glucoside and DIMBOA in wheat (*Triticum aestivum* cv. Millaleu) after inoculation with the obligate parasite *Puccinia recondita*. Control plants (●), infected plants (■). Standard errors are indicated with bars except where included within the data point.

Hx content in roots and leaves

Free AGL was not detected in roots of either control plants or infected plants. However, there was a tendency for GLC levels to be higher in the roots of plants infected with *S. tritici* (1·50 *vs.* 1·08 mmol kg^{-1} fresh wt.; $P = 0{\cdot}065$). GLC content of infected leaves appeared to be lower (0·03 *vs.* 0·12 mmol kg^{-1} fresh wt.) than of control leaves, but due to high variation the differences were non-significant ($P = 0{\cdot}18$).

TABLE 2
Rates of disappearance of DIMBOA-glycoside and DIMBOA from solutions treated with extracts from S. tritici (*ST*) *and* D. teres (*DT*) (*means of two separate tests*)

Treatment	Rate·10^{-3} (min^{-1})	Method
DIMBOA control	1·75±0·04	UV[a]
DIMBOA-GLC+ST extract	0[b]	HPLC[c]
DIMBOA+ST extract	2·45±0·05	HPLC
DIMBOA+ST extract[d]	2·44±0·09	UV
DIMBOA+ST autoclaved extract[d]	1·84±0·04	UV
DIMBOA+ST extract	0·06±0·01	HPLC
DIMBOA+DT extract	0·24±0·02	HPLC

[a]From log A_{263} *vs.* time.
[b]One measurement.
[c]From log area *vs.* time.
[d]Means of two separate extractions of plates 4 and 6 weeks old.

Rates of DIMBOA degradation by fungal extracts

The rates, determined either from changes in HPLC peak area or in absorbance at 263 nm, are given in Table 2. The addition of extracts of *S. tritici* significantly increased the rate of disappearance of DIMBOA from solution. At pH 3·5 all rate-enhancing activity of the extract was inhibited (results not shown).

DISCUSSION

We were able to confirm part of our hypothesis predicting that the rate of hydrolysis of DIMBOA-glucoside in the wheat leaf would depend on the type of fungus involved. When wheat plants were infected by the wheat-compatible necrotroph *S. tritici* the content of GLC decreased in the leaves of both cultivars studied. A week after infection had taken place, the levels in both treated and untreated plants were the same because of the intrinsic decrease of Hx as the plants developed [*25*]. A similar pattern was observed in the treatment with the wheat-incompatible necrotroph *D. teres*, the causal organism of net blotch, of barley. GLC content was lower in the treated plants up to day 4 post-inoculation, after which it became similar to the level in control plants. Finally, as hypothesized, infection with *P. recondita* did not cause GLC hydrolysis. These results may be explained by the different manner in which the fungi penetrate or invade. *S. tritici* and *D. teres* are both facultative necrotrophs, penetrating mechanically and/or enzymatically, growing intra- and intercellularly and disrupting cell membranes, causing osmolysis and leakage of cell contents [*14*, *1*] together with extensive necrosis, depending on the degree of susceptibility of the plant. *D. teres*, a barley pathogen, cannot be expected to provoke the same amount of damage as *S. tritici* because of its incompatible relationship to wheat. Therefore, although penetration and invasion of the leaf, does take place as shown clearly by the increase of total peroxidase activity, growth of *D. teres* is restricted. Consequently, hydrolysis of GLC will be reduced or marginal as found in this study. *P. recondita*, on the other hand is as an obligate parasite and exhibits a different invasive behaviour. Following penetration through the stomata the germ tube develops to form intercellular hyphae from which

haustoria are formed [7]. Present evidence suggest that the more or less continuous interface between the fungus and the host cell includes both an extra-haustorial membrane and the undamaged host plasmalemma. The encapsulated haustorium is separated from immediate contact with host cell protoplasm [7] but nevertheless induces a marked increase in metabolic activity. The unbroken plasmalemma, the non-leaking intact nature of the haustorium, the continuous function of the host cell despite fungal invasion, the distribution of the fungal body in the uppermost leaf cells and also the localization of Hx primarily in the vascular tissues [3], suggest that *P. recondita* is unlikely to release free DIMBOA. This was also found to be the case since the content of GLC did not differ significantly from that of control plants.

The second part of our hypothesis concerned the release of free DIMBOA after cell damage in the leaves, as shown to occur following herbivore feeding [13, 25]. It was anticipated that in the wheat–*P. recondita* interaction no DIMBOA should be produced, for reasons discussed above and this was found to be the case. However, in the case of *S. tritici*, where a significant decrease in GLC content followed infection, no concurrent increase of DIMBOA was observed. This finding could have several explanations, some of which are discussed below.

First, there is the possibility that free DIMBOA is transported away from the site of release, either by phloem loading or passive diffusion. Compounds transported in the phloem include not only assimilates, but also organic acids and phytohormones [32] as well as secondary compounds such as alkaloids [6, 37]. Normally, solutes are first transported from the source leaf to the roots and other metabolically active growing points [32], suggesting that the content of free DIMBOA should be higher in the roots of infected plants than of control plants. As we did not detect any DIMBOA in the roots after *S. tritici* infection this possibility does not seem to occur. We did, however, detect a nearly 40% increase (close to significance) of GLC content in the roots of infected plants. Givovich *et al.* [11] demonstrated that wheat phloem sap collected through aphid stylets contains significant amounts of GLC (up to 4 mM). This raises the possibility that some GLC may be transported unhydrolyzed from a cell damaged by an invading fungus, become loaded into the phloem and translocated to other plant parts, i.e. the roots and growing leaves.

Second, part of the absence of free DIMBOA may be due to *S. tritici* either accumulating or modifying/decomposing it. We have no evidence for the first possibility but, mixing the supernatant of a filtered water extract of *S. tritici* with DIMBOA at pH 6·2 lead to its disappearance at a rate 39% higher than that occurring spontaneously. Mixing DIMBOA with an extract from *D. teres* did not result in higher disappearance rates. This is the first report of an organism being able to increase the decomposition rate of DIMBOA, a compound with high antibiotic activity originally described for its antifungal properties against *Fusarium nivale* [34]. This activity is apparently pH-related as it was totally inhibited at pH 3·5 (results not shown). In none of our analyses did we observe peaks appearing at other retention times or wavelengths which corresponded to 6-methoxy-benzoxazolinone or MBOA, the normal decomposition product of DIMBOA [25]. Wahlroos and Virtanen [34] reported that *F. nivale* was able to decompose MBOA but they did not state the pH nor the temperature at which the reaction took place. However, disappearance of benzoxazolinone was shown to start only after approx. 1 day. Hence, the increased rate of disappearance of

DIMBOA described is not simply due to catalysis of its decomposition by the fungi. Many fungi produce extracellular oxidases [*23*], either constitutively or inducibly, which oxidize deleterious plant phenols. Data are rapidly accumulating showing the capacity of pathogenic fungi to metabolize phytoalexins or other plant defence compounds [*33*]. Thus, Pezet *et al.* [*28*] recently provided evidence that *Botrytis cinerea* Pers. was able to degrade two antifungal phenolic compounds from grape vine, pterostilbene and resveratrol, through oxidative detoxification. The degree of substrate specificity may vary, however, as some fungi may have the capacity to metabolize compounds without being more tolerant to them, or others may be able to metabolize phytoalexins even from non-hosts [*33*]. Our work suggests that the activity shown by the *S. tritici* extract is rather specific towards DIMBOA, since no activity was found against DIBOA, another Hx present in rye (*Secale cereale* L.), or other natural compounds tested (gramine, resorcinol; results not shown). These results would be expected since the fungus is specific to wheat. Moreover, we found that the barley pathogen *D. teres* was unable to alter the rate of disappearance of DIMBOA, which was as expected since it is a non-pathogen of wheat.

We wish to thank Drs R. Madariaga (INIA/Chillán) and R. Cortázar (INIA/La Platina) for help in obtaining fungal isolates and Miss L. Ramírez for technical assistance. The visit to Chile by the first author was made possible by funds from the Swedish Agency for Research Cooperation with Developing Countries (SAREC) and the International Program in the Chemical Sciences (IPICS), both of which are gratefully acknowledged.

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