Alterations in 1,4-Benzoxazinone Levels Following Inoculation with Stem Rust in Wheat Leaves Carrying Various Alleles for Resistance and Their Possible Role as Phytoalexins in Moderately Resistant Leaves

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The contents of 1,4-benzoxazinone derivatives in wheat plants infected with *Puccinia graminis* Pers. f. sp. *tritici* Ericss. & Henn, race 32, and in uninfected controls were examined in four near-isogenic lines of different infection types: *Triticum aestivum* L., cultivar Prelude Sr5 (highly resistant), Sr24, Sr26 (moderately resistant), and srx (susceptible). In all infection types the contents of DIMBOA-glc and HMBOA-glc decrease with time in the uninfected controls as well as in the infected plants. However, following inoculation, the synthesis of HDIBOA-glc is drastically increased in the moderately resistant cultivars. The results suggest that this fully methylated 1,4-benzoxazinone may function as a phytoalexin in this type of interaction. The benzoxazolinone MBOA which has been described as an *in vitro* conversion product of the benzoxazinones mentioned above is not detected in inoculated or uninoculated leaves.

Introduction

1,4-Benzoxazinones have been isolated from various wild and cultivated Gramineae [1]. These cyclic hydroxamic acids have been described as constitutive compounds in the cereals rye, maize and wheat [2–4]. In maize and wheat the main 1,4-benzoxazinone is DIMBOA [1, 5], whereas in rye it is DIBOA, the demethoxylated analogue [2]. Normally these substances are found as glucosides which release the corresponding aglycones upon enzymatic cleavage [6]. Benzoxazinones are thought to be involved in the resistance of cereals to pathogenic fungi and insects [7–10] and also in the resistance of wheat to the rust fungus *Puccinia graminis* [11, 12]. Nevertheless, some inconsistencies with respect to the role of 1,4-benzoxazinones in the resistance of plants to their pathogens remained: The contents of DIMBOA and DIMBOA-glc decrease with age of the leaves in a way that they are no longer detectable in fully grown leaves 3 weeks after germination. But crude and partially purified extracts of the plants are still inhibitory to the germination of *Septoria nodorum* [13]. Furthermore, a negative correlation between the concentration of DIMBOA-glc and the number of rust races attacking the plants was found [14]. This correlation however is entirely due to the two extreme cultivars. Analyzing moderately resistant cultivars the correlation coefficient was found to drop [14].

These contradictory observations encouraged us to further investigate the role of 1,4-benzoxazinones in wheat/rust interactions. Recently, a fully methylated and very labile 1,4-benzoxazinone (HDIBOA-glc) was found in our laboratory to occur in high amounts in *Triticum aestivum* leaves [15]. Here we describe that the content of this compound in wheat leaves increases drastically in response to inoculation with the stem rust fungus.

Materials and Methods

Plants and fungus

Near isogenic lines of the wheat cultivar Prelude (*Triticum aestivum* L.) carrying the stem rust resistance alleles Sr5, Sr24, Sr26 were analyzed. These lines which were kindly provided by R. Rohringer, Agriculture Canada Research Station, Winnipeg, Canada, are classified as follows [16]:

Prelude Sr5, infection type 0 (resistant); Prelude Sr24, infection type 12C (moderately resistant);

Abbreviations: DIMBOA, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one; -glc, β-D-glucopyranosides of 1,4-benzoxazinones; HDIBOA, 2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one; HMBOA, 2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3-one; HPLC, high performance liquid chromatography; MBOA, 6-methoxybenzoxazolinone; TLC, thin layer chromatography.

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Prelude Sr26, infection type 12C (moderately resistant); Prelude srx (background), infection type 3 (susceptible). The stem rust fungus (*Puccinia graminis* Pers. f. sp. *tritici* Ericss. & E. Henn) race 32 was recultured as described [17].

Cultivation of the plants

The plants were cultivated using a slightly modified method of Moerschbacher *et al.* [18]: Seeds were soaked in the dark for 36 h at 25 °C on wet filter paper in glass Petri dishes. Soaked seeds were sowed in soil (type 2, Torfwerk Brill/Neuenhaus, F.R.G.) and grown in a growth chamber under the following conditions: light period 16 h, humidity 60%, temperature 20 °C/18 °C day/night, light intensity 18,000 lx.

Inoculation of the plants

Inoculation of the plants was performed according to Moerschbacher *et al.* [18]. 7 day old plants (including time for soaking the seeds) were inoculated by spraying them with water and subsequently with a suspension of uredospores (1 g of uredospores per 50 ml of 1,1,2-trichlorotrifluoroethane per 325 seedlings) from a distance of 30–40 cm. Prior to inoculation plants of irregular growth were removed. After harvesting the zero-hour sample the inoculated plants were transferred to a plastic tent which was carefully sealed to maintain high humidity conditions. The tents were placed in the growth chamber and kept in the dark for 16 h. Two hours after the onset of the light period the inoculation tent was removed. Controls were treated in the same way with the exception of spraying them only with 1,1,2-trichlorotrifluoroethane.

Extraction and determination of dry weight

Five primary leaves were pulverized under liquid nitrogen and extracted with cold methanol at 0 °C for ½ h. After filtration (Schott Duran, 3D4 sinter glas) the extract was stored at −20 °C until further analysis. To determine the dry weight the residue was dried at 120 °C to constant weight.

HPLC and quantitative determination

Quantitation of benzoxaninones in the crude plant extract was done by HPLC (Milton Roy CM 4000) on a 250 × 4.6 mm column (Kontrosorb 5RP18). The following gradient program was used:

Time [min]	% A	% B	Gradient
0	100	0	
5	95	5	linear
40	85	15	convex (0.1)
70	85	15	
100	80	20	linear
110	0	100	linear
115	0	100	

A = 100% double distilled water with 10 µl H_3PO_4/l l water. B = 100% methanol.

The flow rate was 1 ml/min. Identification of the substances was done by comparing TLC co-chromatography, HPLC peak-enrichment, and absorption spectra with reference compounds prepared as described below. Detection (Milton Roy SM 4000) was carried out at 280 nm. The detector was coupled to an integrator (Milton Roy CI 4000) programmed for automatic quantitation using standard curves obtained with reference compounds.

Preparation of standards

HMBOA-glc, DIMBOA-glc and HDIBOA-glc were isolated from *Triticum aestivum*, cv. 417/65. Primary and secondary leaves of 10 day old plants were pulverized under liquid nitrogen and extracted as described above. After filtration the coloured extract was evaporated to dryness under vacuum at 30 °C. The dried residue was resuspended in a small volume of methanol and chromatographed on thin layer plates (Merck silica gel 60 with 254 nm fluorescent indicator, thickness 0.25 mm). The mobile phase was chloroform/methanol/water (72:25:3). The benzoxazinones were easily detected as dark zones at 254 nm UV light: DIMBOA-glc (R_f 0.29), HMBOA-glc (R_f 0.37), HDIBOA-glc (R_f 0.58). In addition, DIMBOA-glc was rendered visible as a blue spot after spraying the chromatogram with a solution of 2% $FeCl_3$ in methanol/water (1:1). The compounds were eluted from silica gel with a small volume of methanol. The UV spectra were measured and compared with those obtained by Wahlroos and Virtanen [4] and Hofman [19] as well as Hofmann *et al.* [20]. They were found to be identical. When necessary, after pre-

parative TLC the substances were further purified by HPLC as described above.

MBOA was obtained from Calbiochem-Behring Corp., La Jolla, CA 92037, U.S.A.

Results

Using the chromatographic system described above we were able to accurately separate and quantitate MBOA, HDIBOA-glc and DIMBOA-glc/HMBOA-glc directly from the crude extract without any further prepurification. This proved to be a major advantage in the determination of these labile compounds, especially HDIBOA-glc. When using this system, however, it had to be accepted that DIMBOA-glc and HMBOA-glc migrated as one single band and could only be determined as a mixture of compounds. This could be done because of very similar properties of both compounds with respect to the extinction coefficient at 280 nm [21].

The amounts of DIMBOA-glc/HMBOA-glc extractable from the leaves decrease with time. This effect is observed in the infected as well as in the uninfected leaves of all isogenic lines examined. In infected leaves of the cultivar Prelude Sr 24 and srx this decrease is more evident than in uninfected leaves (Fig. 1 a, c). The same results are obtained with Prelude Sr 26 (data not shown). Generally, however, the absolute content of DIMBOA-glc/HMBOA-glc does not depend on the infection type (Fig. 1).

In obvious contrast, the concentration of HDIBOA-glc is strongly increased in the cultivar Prelude Sr 24 as a result of the infection (Fig. 2 a). This effect is visible 3 days after inoculation.

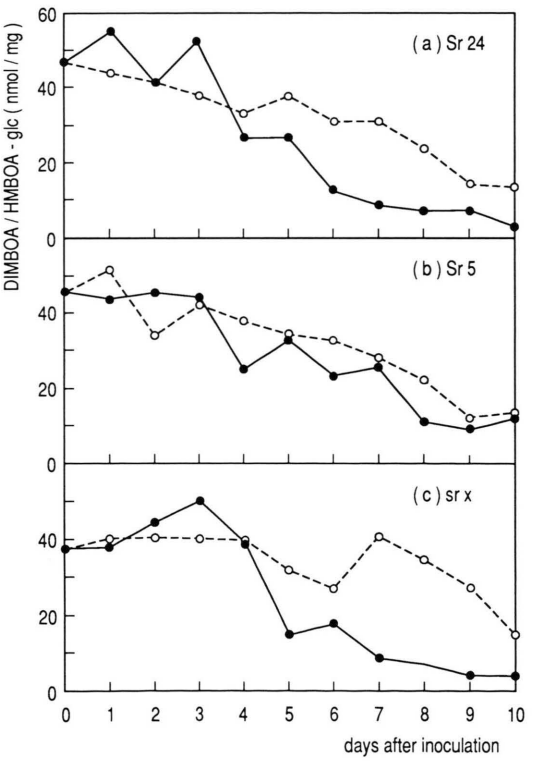

Fig. 1. DIMBOA-glc and HMBOA-glc contents, expressed as amount/dry weight of wheat leaves (nmol/mg) in different near-isogenic wheat lines following inoculation with *Puccinia graminis* f. sp. *tritici*, race 32. (a) Prelude Sr 24, moderately resistant. (b) Prelude Sr 5, highly resistant. (c) Prelude srx, susceptible. ○, Control; ●, inoculated.

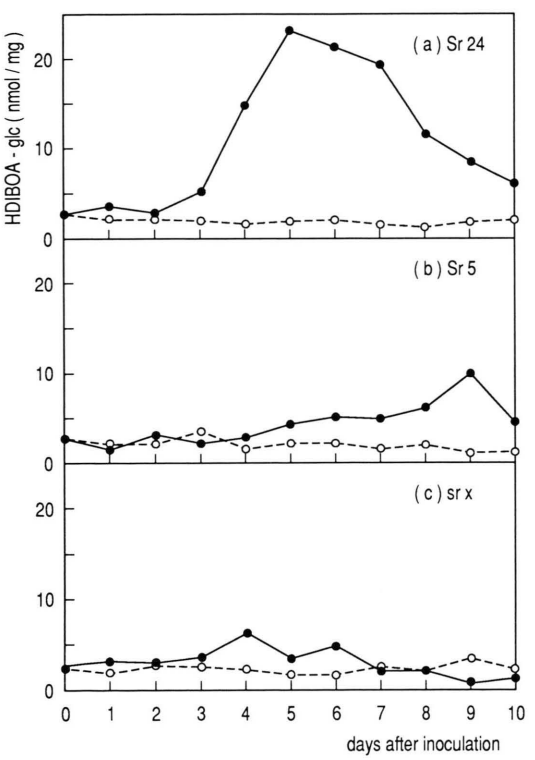

Fig. 2. HDIBOA-glc content expressed as amount/dry weight of wheat leaves in different near-isogenic wheat lines following inoculation with *Puccinia graminis* f. sp. *tritici*, race 32. (a) Prelude Sr 24, moderately resistant. (b) Prelude Sr 5, highly resistant. (c) Prelude srx, susceptible. ○, Control; ●, inoculated.

5 days following inoculation the content of HDIBOA-glc in infected leaves reaches a 10-fold higher level than in the control. The amount of HDIBOA-glc in uninfected plants does not vary (Fig. 2). Very similar results are obtained with the cultivar Prelude Sr26 (data not shown). It should be pointed out that this effect is only observed in the lines carrying alleles for moderate resistance. In highly resistant and susceptible plants the level of HDIBOA-glc remains low (Fig. 2b, c).

The benzoxazolinone MBOA can neither be detected in the infected nor in the uninfected plants. This result is obtained for all lines examined.

Discussion

The glucosides of 1,4-benzoxazinones are found in a number of gramineous plants. Free aglucones are not detected in uninjured plants provided that precautions are taken during extraction. Therefore the resistance of plants to the pathogen cannot be attributed to the presence of free aglucones but it may correspond to the high content of the glucosides and the capacity to release the aglucones by enzymatic cleavage [6]. *In vitro,* free 1,4-benzoxazinones spontaneously decompose to the highly toxic benzoxazolinone MBOA. This is especially true for the extremely labile HDIBOA which after release from the corresponding glucoside is rapidly converted into MBOA and which, so far, could not be purified [15].

The contents of DIMBOA-glc/HMBOA-glc decrease in infected and uninfected plants with time. This is in accordance with results obtained by Argandoña and Corcuera [22] who found the concentrations of cyclic hydroxamic acids in maize leaves to decrease by one third form 7 day old to 20 day old plants. We observed no correlation between the absolute amounts of these compounds and the infection type of the four near isogenic lines examined.

HDIBOA-glc has not been the subject of special interest in the past. Probably due to its instability it was not found in earlier studies. We observed that it, indeed, may decompose to some degree during conventional isolation and purification techniques such as extraction with subsequent evaporation of the solvent (unpublished results).

The content of HDIBOA-glc varies significantly in the infected wheat lines. In the moderately resistant lines Prelude Sr24 and Prelude Sr26 the formation of HDIBOA-glc is strongly induced by the infection, whereas in the resistant and susceptible lines the content does not differ between the infected and uninfected plants. This induction of HDIBOA-glc in the moderately resistant plants which is detectable 3 days, and highly significant 4 days, following inoculation correlates well with the growth of the fungus in these lines. With the beginning of the fourth day after inoculation the mycelial growth in Prelude Sr24 is clearly inhibited in comparison to the growth in the susceptible plants [16].

On a thin layer chromatogram of concentrated plant extract uredospore germination of *Puccinia graminis* is completely inhibited by HDIBOA-glc. It may well be envisaged from the results that *in vivo* a critical concentration for inhibition may easily be reached at affected host cells.

The presence or absence of the decomposition product MBOA in uninjured maize tissue has been the subject of controverse discussions. While its occurrence has been reported by some group [23] others argued that such an observation could be an artifact [24]. In our investigation we did not unequivocally find MBOA in the extracts of uninfected or infected wheat leaves. But this does not exclude the possibility that critical amounts of this highly toxic compound which inhibits germination at concentrations of 10^{-6} M are formed at the infection site. After extraction and, as a consequence, dilution to extremely low concentrations, MBOA may have escaped detection by our techniques used. It should be mentioned that MBOA was readily produced from HDIBOA-glc when the benzoxazinone was brought into contact with isolated fungal cell walls which apparently contain β-glucosidase activity (unpublished results). From our results it is obvious that HDIBOA-glc can not be regarded as an end product in metabolism because of its decreasing concentration at later stages of the infection. It is also clear, however, that MBOA can not be the principal compound of HDIBOA metabolism in leaves because MBOA would have been detected if the decrease of HDIBOA-glc concentration resulted entirely in the formation of MBOA.

There have been a few reports only on the occurrence of phytoalexins in gramineous plants ([25], review article). Examples include the momi-

lactones and oryzalexines which have been described as inhibitory tricyclic diterpenoids in infected rice plants [26, 27] and the avenalumins in oats which on a speculative basis are derived from anthranilic acid and are thus biochemically related to the 1,4-benzoxazinones. Avenalumines accumulate in oats infected with the crown rust fungus directly at infection sites or in leaves treated with various elicitors [28].

So far, any effort to identify induced inhibitory compounds in wheat leaves had failed [29]. Instead, strong arguments were presented that lignification is an important defensive response in highly resistant wheat plants (infection type 0) [30, 31]. In conclusion, our results indicate that 1,4-benzoxazinones play a role in wheat plants of a moderate infection type carrying the Sr24 or Sr26 allele. HDIBOA-glc (but not HMBOA-glc or DIMBOA-glc) may have to be regarded as a phytoalexin.

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