Compounds of natural origin inducing winter wheat resistance to powdery mildew (*Blumeria graminis* f.sp. *tritici*)

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ABSTRACT

In laboratory and small-field experiments inducers of synthetic origin: benzothiadiazole (BTH), salicylic acid, and inducers of biological origin: glycine betaine, extracts prepared from oak bark (*Quercus robur* L.), *Reynoutria sacchaliensis* L., curcuma (*Curcuma longa* L.), ginger (*Zingiber officinale* Roscoe) were effective against powdery mildew on the winter wheat (cv. Kanzler) susceptible to this disease. All studied inducers slightly effected the synthesis of new proteins (PR-proteins) that were localized in extracellular space. The efficacy of inducers was long-term. The most effective inducer was BTH; its application produced a number of chlorotic blotches on leaves.

Keywords: winter wheat; inducer; powdery mildew; new proteins; disease severity

Plants have developed multiple defence strategies against colonization by microbial pathogens. A natural protection of plants against pathogens or insects is based on different mechanical and chemical barriers that exist in plants before current infection. Resistance to diseases is a genetically based ability of a plant to stop or to reduce the activity of a pathogen, which can have lots of forms (Goodman et al. 1986). Two types of resistant reactions can be distinguished: non-host and host or race cultivar specific resistant reaction. In both cases biochemical processes included in resistance to a pathogen are very similar (Somssich and Hahlbrock 1998). Induced resistance covers two categories: systemic acquired resistance and systemic induced resistance (Hammerschmit 1999).

The aim of the present study was to investigate the effect of chosen biological and synthetic inducers on disease severity of powdery mildew (*Blumeria graminis* f.sp. *tritici*) and physiological changes, namely PR-protein accumulation in winter wheat.

MATERIAL AND METHODS

Plants, pathogen, and design of experiments

Two experiments, laboratory and small-plot, were done on winter wheat (*Triticum aestivum* L.), variety Kanzler (susceptible standard to powdery mildew). Treatment of plants with inducers was made by means of small hand sprayer.

In laboratory experiments (2003) young plants of winter wheat were grown in pots in the growth chamber (photoperiod 16 hours, temperature 18°C, light 15 000 lux). Inducers were applied on wheat plants on the 20th–23rd day after emergence. The cultivar Kanzler was treated with inducers one week before inoculation by powdery mildew. A mixture of races virulent to genes of resistance Pm2, 6; Pm4b was used as inoculum. Leaf segments (Flath and Andersch 1996) were prepared from the first leaves of Kanzler 7 days after the treatment by inducers. Segments were transferred on water agar with contents of 80 ppm of benzimidazole in plastic boxes. Boxes were placed under settle tower and inoculated by conidia of powdery mildew *Blumeria graminis* f.sp. *tritici*. Induced resistance was determined according to disease severity that was evaluated after 12 days by a nine-point assessment scale (Flath and Anders 1996). There were made treatments with seven inducers and one untreated control in four replications.

In small-plot experiments (2004) the cultivar Kanzler was used, too. Infection of powdery mildew was natural. The same inducers were used as in the laboratory experiments. Two rows of the cultivar were treated on 7.6.2004 in the

growth stage 49. Between the two treated rows three rows were left without treatment from both sides. The disease severity of leaves was scored in five sampling dates by evaluation of all live leaves on 10 plants in two replicates by a nine-point assessment scale (Saari and Prescott 1975). From the disease severity data the percentage of each leaf and the cumulative proportion of leaf area disease – CPLAD were counted (Věchet and Kocourek 1987, Brière et al. 1994). Programs MS Excel (MICROSOFT, Corp., USA) and STATISTICA (STATSOFT, Inc., TULSA, USA) were used for statistical evaluation. The growth stage was assessed using Zadoks decimal code (Zadoks et al. 1974).

Preparation of inducers

Inducers were prepared as water solutions (BTH, SA, glycine betaine), or water plant extracts as follows: benzothiadiazole (BTH) – 1.2mM, salicylic acid (SA) – 1mM, glycine betaine – 0.3M; extracts from curcuma – (*Curcuma longa* L.) – CU, ginger (*Zingiber officinale* Roscoe) – GI, *Reynoutria sacchaliensis* L. – RS, preparation from oak bark (*Quercus robur* L.) – OB.

Rhizomes of curcuma and ginger, respectively, were grinded in distilled water (2 g + 10 ml) using pestle and mortar. The homogenate was filtrated through miracloth and used immediately for spraying the plants.

Dry leaves of *R. sacchaliensis* (0.5 g) were grinded in mortar to prepare powder and kept stirring for 30 min in distilled water (25 ml) on magnetic stirrer. The suspension was filtrated through miracloth and used for treatments.

The preparation of oak bark was dispersed in distilled water (0.067 g/10 ml), kept stirring for 30 min, filtrated through miracloth, and sprayed on plants. In laboratory experiments, the oak bark preparation was also added to cultivation substrate (0.102 g) and mixed well.

Extraction of extracellular fluid and protein analysis

Extracellular proteins were extracted by means of vacuum infiltration followed by low-speed centrifugation at 1000 g as described by Pierpoint (1987). Proteins present in a washing fluid were analyzed using native polyacrylamide gel electrophoresis (PAGE), the gels were silver stained (Hames and Rickwood 1990).

RESULTS

Laboratory experiments

All used inducers reduced the percentage of disease severity of powdery mildew (Figure 1). The least effective was salicylic acid (78% of the control), the highest effect showed benzothiadiazol (14% of the control) and extract of curcuma (25% of the control).

Statistic evaluation

The identification of significant pairs of inducers averages on emergence of SAR was carried out by analysis of variance (Table 1).

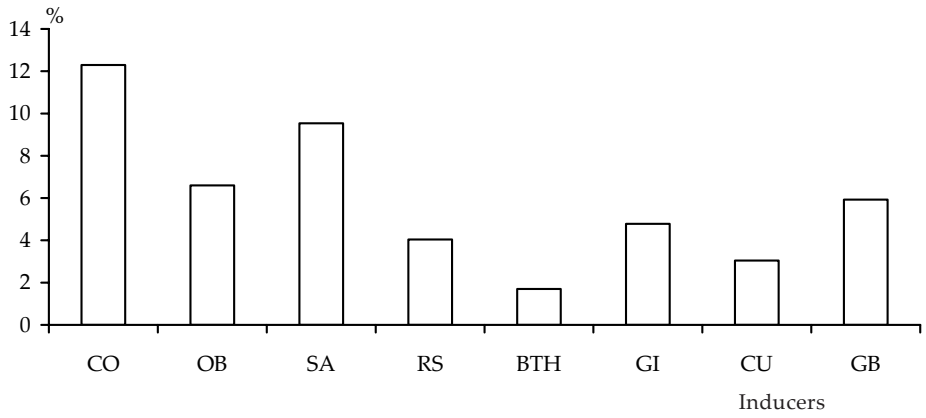

Figure 1. Percentage of disease severity of powdery mildew on winter wheat, cultivar Kanzler

control – untreated; inducers: OB – oak bark (0.067 g/10 ml), SA – salicylic acid (1mM), RS – extract of *Reynoutria sacchaliensis* L., BTH – benzothiadiazole (1.2mM), GI – extract of ginger, CU – extract of curcuma, GB – glycine betaine (0.3M)

Table 1. Duncan test; OB – oak bark, SA – salicylic acid, RS – *Reynoutria sacchaliensis* L., BTH – benzothiadiazole, GI – ginger, CU – curcuma, GB – glycine betaine; differences among means with probability p = 0.95 are highlighted bold

Duncan test
Probabilities for Post Hoc Tests
Main effect: var. 2

	Control	OB	SA	RS	BTH	GI	CU	GB
Average	***12.16667***	***6.500000***	***9.500000***	***4.000000***	***1.666667***	***4.750000***	***3.000000***	***5.833333***
Control								
OB	**0.000009**							
SA	0.057698	**0.032745**						
RS	**0.000004**	0.105068	**0.000218**					
BTH	**0.000005**	**0.001541**	**0.000004**	0.117081				
GI	**0.000005**	0.242574	**0.001347**	0.593472	**0.043421**			
CU	**0.000004**	**0.023996**	**0.000014**	0.476623	0.342619	0.242574		
GB	**0.000015**	0.635144	**0.012373**	0.220521	**0.006352**	0.440674	0.064679	

Induction of PR-proteins

The induction of new extracellular proteins was analyzed by means of native PAGE. Apart from BTH, all inducers caused only a slight induction of PR-proteins or an increased amount of already present ones (Figure 2). The most efficient inducers were BTH and ginger extract.

Small-plot experiments

The evaluation of an effect of inducers was done (Table 2) in five sampling dates (SA). All inducers reduced disease severity of powdery mildew (Figure 3). Differences between untreated control and variants treated by inducers were evident in all sampling dates. Disease severity in control continuously rose (from SD1 up to SD5), whereas once in the case of variants treated by inducers rose from SD1 up to SD2 in RS, GI and CU and then stopped or continued slightly up to SD3 such as in OB and GB or exceptionally up to SD4 in BTH, SA and then it stopped. Their effectiveness (Figure 3) was always successful in different terms after application (26, 34, 40 and 54 days). The most effective on the whole plant was BTH at all sampling dates. Considerable effect had GI, CU, GB and also RS even if the effect of BTH was significantly higher than the effect of other named inducers. Lower effect but low disease severity in comparison with control was found in OB and SA.

Differences between disease severities of single leaves were found (Table 3). The highest disease severity was detected in all leaves on untreated control plants in comparison with treated variants. The highest severity was found on the third leaf followed by the fourth, the second and the first leaf from the top. After the treatment with the inducers the fourth leaves displayed the highest disease severity and the third, the second and the first leaves followed with downward severity. Only after the treatment by BTH the fourth leaf in the cultivar Kanzler was dead. Percentage proportion of disease severity (PCO) on the first leaf treated by inducers to untreated control was the highest of other leaves. Only in BTH, GI, GB was lower than 50% and in the other cases PCO was higher than 50% of the control. On the contrary, on next leaves of the plant (the second and the third), PCO was always lower than 50% of the control. Similarly as the first leaf PCO on the fourth leaf after the treatment by SA, OB and RS was higher than 50% of the control. In SA PCO reached not less than nearly

Table 2. The sampling dates (SD) and the corresponding averaged Zadoks growth stage (GS) values for winter wheat cultivar

SD	GS
SD1 – 7. 6.	53
SD2 – 14. 6.	65
SD3 – 22. 6.	69
SD4 – 28. 6	71
SD5 – 12. 7.	75

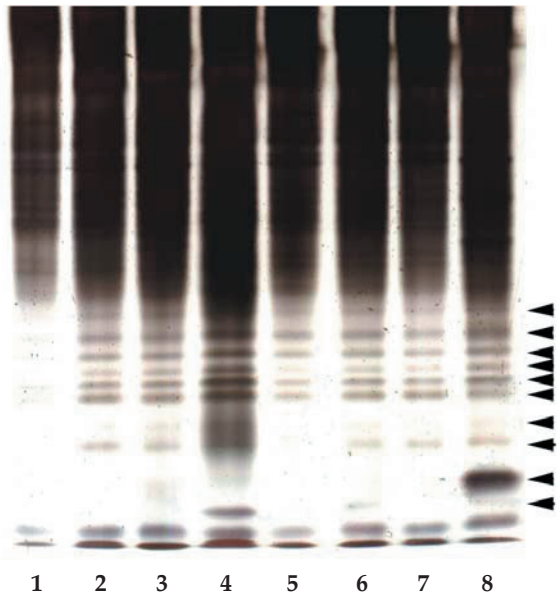

Figure 2. Extracellular fluid recovered from wheat extracellular spaces was analyzed 7 days after induction using native PAGE

The order of samples: 1 – control, 2 – salicylic acid (1mM), 3 – oak bark (0.067 g/10 ml), 4 –extract of ginger, 5 – extract of curcuma, 6 – extract of *R. sacchaliensis* L., 7 – glycine betaine (0.3M), 8 – BTH (1.2mM); increased content of extracellular proteins in inducer-treated plants in comparison with control is shown by arrows

96% of the control. The highest effect on the third leaf was visible after the treatment by RS, BTH and GB. On the contrary, the effect on the second leaf from the top was obvious after the treatment by OB, SA, GI and CU. After application of BTH tiny chlorotic blotches were visible on leaves.

DISCUSSION

Inducers used in these experiments showed an effective protection against powdery mildew on wheat.

Laboratory experiments showed that although salicylic acid and BTH induced extracellular proteins their effect on induction of resistance was different. BTH effectively induced the resistance to powdery mildew. Görlach et al. (1996) and Stadnik and Buchenauer (2000) obtained similar results. Induction of this resistance can be related to production of PR-proteins with chitinase and glucanase activity. Application of salicylic acid decreased disease severity of powdery mildew only to 78% in comparison to the control, while BTH reduced disease severity to 14%. Van Loon and Pieterse (2002) found out that salicylic acid is endogenously important for emergence of systemic acquired resistance but leaf application might not induce this phenomenon. The ability of glycine betaine to induce resistance to pathogens has not been studied yet, however its capability of inducing both chitinase and β-1,3-glucanase in sugar beet roots and leaves was reported (Burketová et al. 2003/2004). In our experiments, glycine betaine significantly reduced disease severity of powdery mildew in wheat. Extracts from *Reynoutria sacchaliensis* L. are registered as Milsana® (Münster, Germany) and they are used for protection against powdery

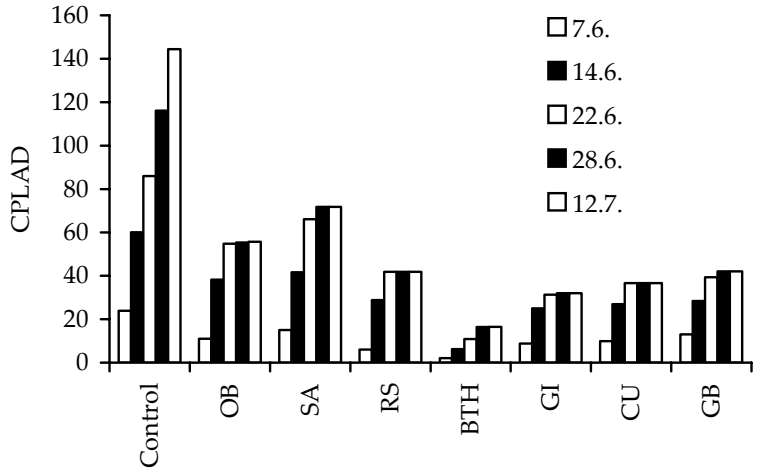

Figure 3. Disease severity of powdery mildew (CPLAD) in the small-plot experiments on all leaves cv. Kanzler

control – untreated; inducers: OB – oak bark (0.067 g/10 ml), SA – salicylic acid (1mM), RS – extract of *Reynoutria sacchaliensis* L., BTH – benzothiadiazole (1.2mM), GI – extract of ginger, CU – extract of curcuma, GB – glycine betaine (0.3M); the date of inducers application: 19.5.2004, the date of evaluation: 7.6., 14.6., 22.6., 28.6., 12.7.2004

Table 3. Maximum of disease severity of powdery mildew on individual leaf according to its position on wheat plant cv. Kanzler 2004

Inducer	1. leaf	PCO	2. leaf	PCO	3. leaf	PCO	4. leaf	PCO
CO	3.45	–	27.15	–	62.50	–	51.25	–
OB	1.90	55.07	4.05	14.92	13.30	21.28	36.40	71.02
SA	2.20	63.77	4.25	15.65	16.20	25.92	49.05	95.71
RS	1.25	36.23	4.80	17.68	9.20	14.72	26.50	51.71
BTH	0.45	13.04	4.95	18.23	11.10	17.76	+	+
GI	0.85	24.64	4.80	17.68	4.80	7.68	16.95	33.07
CU	2.10	60.87	5.70	20.99	5.70	9.12	11.00	21.46
GB	0.85	24.64	4.80	17.68	16.15	25.84	19.65	38.34

Expressed as percentage of each leaf covered by powdery mildew and CPLAD – percentage of each leaf and the cumulative proportion of disease of all leaves on single leaves untreated – CO and treated by inducers; the flag leaf is the first from the top; + already dead leaf; PCO – percentage proportion of disease severity of used inducers to untreated control

mildew on cucumbers (Daayf et al. 1997, Würms et al. 1999). Here extracts from *Reynoutria sacchaliensis* L. induced resistance to powdery mildew on wheat. Substances present in curcuma and ginger had antifungal (Miah 1990) and antiviral activity (Šindelářová et al. 1996). Water extracts of ginger and curcuma also induced the resistance to powdery mildew effectively. The effect of biodynamic preparation from oak bark, which is an alternative method of plant protection, was interesting. In spite of a very low concentration of this preparation it induced resistance to powdery mildew. Nevertheless, the extract from oak bark similarly to extracts from curcuma, ginger and *Reynoutria sacchaliensis* L. induced new PR-proteins only slightly, which indicates that different mechanisms probably took place in this particular case of resistance.

For reduction of disease severity in small-plot experiments the most effective was BTH (Bion ®) but it seems that the oldest leaf (the fourth leaf from the top) died earlier than in the case of other inducers. Application of BTH was remarkable by a large number of chlorotic splotches on most leaves and on the oldest leaf (the third from the top) necrotic ones. Besser et al. (2001) found out that the treatment of susceptible barley plants with Bion® activates resistance against *Blumeria graminis* f.sp. *hordei*. Görlach et al. (1996) showed that the onset of resistance in wheat after application of BTH was accompanied by the induction of a number of newly described wheat chemically induced (WCI) genes, including genes encoding a lipoxygenase and sulfur-rich-protein. In our experiment the treatment by ginger, curcuma, glycine betain, and *Reynoutria sacchaliensis* was very effective. Slightly less effective results were reached after treatment with oak bark and salicylic acid. Differences between the untreated control and the application of inducers were most obvious on the oldest (fourth) leaf. Important is the fact that action of inducers was long-term. Disease severity of powdery mildew on the first leaf (flag leaf) was the smallest from all leaves on the plant. On this leaf differences between untreated control and variants treated with single inducers was not so significant as on other leaves. Disease severity in whole plants treated by inducers (expressed as CPLAD) was lower than 50% of control except salicylic acid. Similarly, the second, the third and the fourth leaves had disease severity under 50% of the control except the second leaf after treatment by curcuma. It is possible to use another inducers of resistance. For example Kogel et al. (1994) found out that treatment of susceptible barley (*Hordeum vulgare*) seedlings with 2,6-dichloroisonicotinic acid (DCINA) induces disease resistance against the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*). Similarly Besser et al. (2000) stated that salicylic acid (SA) and its synthetic mimics 2,6-dichloroisonicotinic acid (DCINA) and benzothiadiazole (BTH) protect barley systematically against powdery mildew (*Blumeria graminis* f.sp. *hordei*) infection by strengthening plant defence mechanisms that results in effective papillae and host cell death. In our case very interesting was long lasting effect, i.e. fifty-four days after their application.

All used inducers incited resistance to powdery mildew. Our results prove that chemically induced resistance in plants leads to a durable protection

against powdery mildew and indicated that even biological inducers could provide long-term protection against powdery mildew in wheat.

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ABSTRAKT

Směsi přírodního původu indukující rezistenci ozimé pšenice k padlí travnímu (*Blumeria graminis* f.sp. *tritici*)

Proti padlí travnímu na náchylné odrůdě ozimé pšenice (odrůda Kanzler) byly v laboratorních a polních pokusech použity efektivní induktory syntetického původu: benzothiadiazole (BTH), kyselina sallicylová a induktory biologického původu: glycine betain, extrakty připravené z dubové kůry, křídlatka sachalinská, kurkuma, zázvor. Všechny studované induktory lehce indukovaly syntézu nových proteinů (PR-proteinů), které byly lokalizovány v extracelulárním prostoru. Nejvíce efektivním induktorem bylo BTH. Účinnost induktorů byla dlouhodobá. V důsledku aplikace BTH se tvořily četné chlorotické skvrny na listech.

Klíčová slova: ozimá pšenice; induktor; padlí travní; nové proteiny; intenzita choroby

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