BIOCHEMICAL FEATURES OF WINTER WHEAT GRAIN AFFECTED BY BIOLOGICAL AND CHEMICAL CONTROL TREATMENTS*

Urszula Wachowska1, Iwona Konopka2, Małgorzata Tańska2, Ewa Korzeniewska3

1Department of Entomology, Phytopathology and Molecular Diagnostics
2Department of Food Plant Chemistry and Processing
3Department of Environmental Microbiology
University of Warmia and Mazury in Olsztyn, Poland

Abstract

A new trend in plant protection consists in the integration of biological and chemical control treatments. Unfortunately, the biological control agents for winter wheat are still in short supply. Bacteria of the genus *Sphingomonas* have a unique ability to produce prolyl endopeptidases. Those enzymes are capable of hydrolyzing the peptide PQPQLYPQPQLP. During the growing season, the bacteria may be used to protect winter wheat against infections caused by fungi of the genera *Fusarium*. The objective of this study was to evaluate the effectiveness of bacterial isolates in protecting field-grown winter wheat plants against spike infections and to assess the effect of bacteria on the chemical composition and microbiological purity of winter wheat grain. The effects of bacteria of the genus *Sphingomonas* as biological control agents against *Fusarium* head blight (FHB) of winter wheat were evaluated in a three-year field experiment. For comparative purposes, the fungicides propiconazole at the elongation stage (BBCH 31) and fluoxastrobin + prothiconazole at the heading stage (BBCH 55) were applied. In 2010 and 2011, the application of cell suspensions of bacteria alleviated the symptoms of disease by 27.3% and 75.8%, respectively in comparison with control. Wheat grain yield was higher in plots subjected to the biological and chemical treatment (by an average of 9.5 and 13.6%, respectively). For the first time, we observed that biological control modified the chemical characteristics of wheat grain. In control grain, the content of gluten proteins was 7.9% higher than in grain treated with the biocontrol agent. In wheat grain treated with the biocontrol agent, the highest decrease was observed in the concentrations of alpha/beta-gliadins (10.59%), but grain quality was most affected by an estimated 8% decrease in the content of HMW glutenins. Biological treatment inhibited the growth pathogens of *F. culmorum*, *F. poae*, *F. sporotrichiodes* and *F. avenaceum*. A cell suspension of bacteria did not inhibit the growth of yeasts and epiphytic bacteria of the genus *Azotobacter* on grains.

Keywords: biological control, Sphingomonas sp., protein, microbiota, Fusarium spp.
INTRODUCTION

Biochemical characteristics of wheat grain could be changed by a difference of phosphorus and potassium fertilization level, pathogenic infections and various chemical treatments (Gaj et al. 2013, Rodrigo HW DO 6|PSWRPVR |IXVDULXPKHDEO|L|KW |+|DUH REVHUYHGRQZKHDV|SLNHV |LQ HSLGHPLF|HDUV7|KRV|SDWKRJHQVF RQWDPLQDWH|JUDLQ|ZLWK|P|FRWR|LQV|DOG the predominant metabolites accumulated in wheat grain infected by F. culmorum and F. graminearum DUHGHRLQ YDOHQR'O21DOQRWKHUWULFKRWKH cenes as well as zearalenone (Stepień, Chełkowski |IHFWLHQHVRII synthetic fungicides in inhibiting the proliferation of Fusarium fungi is often OLPLWHGDQGWKHLQQRWUHGXFHWR|LQFRQHQWUDWLEQVUQJUDLQ still in short supply. During the growing season, bacteria have been used to protect winter wheat against infections caused by fungi of the genera Fusarium (Schisler HW DO Khan, Doohan 2009, Wachowska et al. 2013). Bacteria of the genus Sphingomonas have a unique ability to produce prolyl HQGRSHWLGDHVZKLFKKGURO|HWKHSWSLGH ERQQRW KHDUER[|OVLGH R a proline residue (Kabashima HWDO7KHVHHQ)|PHVDUHFSDSEOHR|IK|GUR lyzing the peptide PQPQLPYPQPQLP, and they have been proposed as oral treatment for celiac disease (Osorio et al. 2012). The objective of this study ZDVHYDONDXDWHWHKHHIIHFWLYHQHVVRIEDFWHULDOLVRODWHVLQSURWHFWLQH winter wheat plants against spike infections, and to evaluate the effect of bacteria on the chemical composition and microbiological purity of winter wheat grain.

MATERIAL AND METHODS

Potential biocontrol agents

ORVVLVRODWHVLQHWHGDLVHLQGDLVHQRQVHTXHQFHVRKLGHQWLHGEDVHG RQVHTXHQFHVRIWKH76U1$UHJ.LR(Wachowska et al. 2013). The source of carbon and the enzymatic activity of isolates were deter-PLQHGLZLWKHKHS31PFLURWHVVW|VWHPELR0PULHX|LQDFRUGDFHZLWK the manufacturer's instructions. Bacterial isolates used in biological control WUHDWPHQWVZHUHLGHQWHLGDEVHORQJLQJWRWKH genus Sphingomonas666662ZHUHDJDPQHZ|DWLYH7KUHPDL
ning isolates of *Sphingomonas* sp. assimilated most tested substrates and reduced nitrates to nitrogen.

**Preparation of bacteria cell suspensions for biological control treatments**

Bacterial suspensions were prepared twice in each winter wheat growing season. Isolates were cultured for seven days in Petri dishes on solid potato dextrose agar (PDA) medium with pH 7.2, in the dark, at 27°C. After incubation, bacterial colonies were removed from the medium (50 Petri dishes for each isolate) with an inoculation loop and placed in 100 cm³ sterile water.

7KHEDFWHULDQVXSVQVRLQZDVDP[LWXUHLIRVORDHWVZLKHFOOQHGVLLW\IR \CFU per 1 cm³ water. The suspension was applied to plants at the stem elongation stage (BBCH 31) and the heading stage (BBCH 55) (Meier 2003). Prior to application, one liter of bacterial cell suspension was diluted in 10 dm³ water and sprayed over an area of 80 m² (four plots of 20 m² each).

**Field experiment**

$_{\text{HOG H}}[\text{SHULPHQW ZDV HVWDEOL VKHG DW WKH H[SHULPHQWDO VWDWLP}}$

7RPDV)NZRE0LQ 7KHH[SHULPHQW KGDUDQGR -P[GEORFGHVL]QZLWKIRXUHSOLFDWLRQ[VQWHU]K(H "cv. Bogatka, a bread wheat cultivar, was sown in 20 m² plots. Control plots were sprayed with water twice. For comparative purposes, the fungicides %XPSHU (SRULFRQD)ROH DQG )DQGDOR (XR[DVWUREQ DQG prothiconazole) were applied at the stem elongation stage (BBCH 31) and the KHDGLQJ[VWDJH%+%&+HVSHFWLYHO7KHIQXQJLFLGHVZDHHXVG DWDW KHDQX facturer’s recommended dose of 1 dm³ ha⁻¹. Plants were fertilized with nitrogen (N) at 100 kg ha⁻¹, potassium (K) at 70 kg ha⁻¹ and phosphorus (P) at N\text{J}K D⁻¹.

**Evaluation of the health status of spikes and grain yield**

7KHLQWHQVLW\IR)++ZDV[H[SUHVHLQWHPVRIWKHHDYHUD]HSHUFHGW\DH DUHDIR VSLSNH VKRZQLQV\PSWPRVRIWKHDQDO]HGGLVHVDHV 6SLNHV ZDV HYDOXDWHGQWKHDZW\UULSVWHDJH%+%&+:KHDW ZDV KDUHV WHG DW WKH IXOO\ULSHVWDW\HURPDQDUHDRP with a plot combine. Yield was determined as the weight of grains harvested from an area of 1 m².

**RP-HPLC analysis of wheat grains proteins**

7KUHH SURWHLQ IUDFWLRQ ZHUH [WUDFWHG IURPSXULH[G JUDLQV JURXQGW particle size of 300 mm: 1) albumins and globulins, 2) gliadins, 3) glutenins. 7KHH[WUDFWLRQ SURFHVV ZDV SHUIRUPHG ZLWK WKH XVHRIVROYHQWV GHVFULEH WIESER et al. (2000). For the determination of protein content, grain was harvested in 2010 from control and protected plots. It was ground to produce ||RXU ZLWK WKH SDOUFLQH VLOH W\H EHR\Q \B 7K SURWHLQ FRQWHQW DQG FRPSR sition were determined by the RP-HPLC technique described by KONOPKA.
et al. (2007), at the following parameters: a RP-18 Vydac 218TP54 column with 5\(\text{m}\) bead size and 300 Å pore size, 250 x 4.6 mm; a Zorbax 300SB-C18 pre-column, 4.6 x 12.5 mm; a column temp. of 45\(\degree\)C, a mobile phase flow rate of 1 cm\(\text{min}^{-1}\), and an injection volume of 20\(\mu\)l. A two-component gradient was used. A component: 0 min 75\%, 5 min 65\%, 10 min 50\%, 17 min 25\%, 18 min 15\% and 19 min 75\%. The first component (A) was water with 0.1\% of TFA and the second (B) was ACN with 0.1\% of TFA. The absorbance spectra of eluted proteins were determined by a diode-array detector (HP 1050). Quantification of proteins was done by UV absorbance at 210 nm. The integration procedure was performed using HPLC 3D ChemStation software. The content of every protein fraction was expressed in terms of peak area (mAU x s) per mg of grain. Proteins were detected at 210 nm wavelength. Bovine serum albumin (BSA) manufactured for Bio-Rad (Bradford Protein Assay) and gliadins isolated from the grain of wheat were used as the standards.

Evaluation of microbiological grain features

Grain samples for microbiological analyses were obtained immediately DIWHU WKH UHV LVQ 10 g were placed in 250 cm\(^3\) flasks filled with 90 cm\(^3\) sterile water. The flasks were shaken for 30 min on a shaker table (358 S). Microbial suspensions were twice diluted: pseudomonads – at 10\(^{-3}\) and 10\(^{-4}\), and the remaining microorganisms – at 10\(^{-1}\) and 10\(^{-2}\). Cell suspensions at two dilutions were cultured by the pour plate method in Petri dishes.

Statistical analysis

An analysis of variance (ANOVA) was performed using Statistica 12.0 software. The significance of differences in wheat yields and in the content of protein fractions between treatments was determined by the Student-Newman-Keuls test. Bacterial and fungal colony forming units were counted on Petri dishes. The structure of fungal communities was described as the number of colonies identified on Martin's medium.

The results were log transformed according to the log(CFU+1) formula, and the significance of differences between means was evaluated by the Student-Newman-Keuls test.
RESULTS AND DISCUSSION

In 2010 and 2011, when the severity of FHB was low, the application of cell suspensions of bacteria alleviated the symptoms of disease by 27.3% and 75.8%, respectively, in comparison with control (not significantly) – Table 1. Khan and Doohan (2009) demonstrated that selected bacterial isolates of *Pseudomonas fluorescens* inhibited the progression of FHB by 44% in field-grown wheat inoculated with *F. culmorum*. In our studies, treatments involving a mixture of bacterial isolates of the genus *Sphingomonas* were more effective than Fandango 200 EC. Likewise, Schisler et al. (2006) observed that under field conditions, pseudomonads isolated from wheat (*Pseudomonas* sp. AS 64.4) reduced the severity of FHB more effectively than the Folicur 3.6 F fungicide (tebuconazole).

In successive years of the experiment, biological control increased winter wheat yield by 2.3%, 3.4% and 18.6% (not significantly), respectively, as compared with the control treatment (Table 1). A significant average increase in grain yield was observed in treated plots during the three-year experiment. In plots treated twice with fungicides, yield increased by 5.3%, 8.9% and 22.8% in successive years of the study, respectively, in comparison with control, but the noted increase was significant only in 2011.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Year</th>
<th>Severity of Fusarium head blight mean</th>
<th>Grain yield from 1 m² mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2009</td>
<td>2.31a</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>0.11b</td>
<td>422.7a</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>0.33b</td>
<td>c</td>
</tr>
<tr>
<td>Bacteria</td>
<td>2009</td>
<td>4.40a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>0.17b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>0.30v</td>
<td>x</td>
</tr>
<tr>
<td>Fungicides</td>
<td>2009</td>
<td>3.08x</td>
<td>501.56</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>0.12y</td>
<td>440.10z</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean values assigned the same letter differ not significantly within columns according to SNK-test at p < 0.01 (a - g – means for years and pathogens, x-z – means for years).
Albumins and globulins accounted for 18.3% of wheat grain proteins on average (Table 2). The remaining 81.7% proteins were gluten proteins, mostly gliadins which had a 64.2% share of that fraction. Gliadins consisted mostly of alpha/beta-gliadins as well as gamma- and omega-gliadins with a 55.5%, 33.1% and 11.4% share, respectively. In the glutenin fraction, aggregates of high molecular weight (HMW) accounted for 30.8%. The analyzed plant protection agents lowered the content of most protein fractions. The only exceptions were omega-gliadins and low molecular weight (LMW) glutenins, whose concentrations remained constant. In control grain, the content of gluten proteins was 5.22% higher than in fungicide-treated grain and 7.9% higher than in grain treated with the biocontrol agent. In wheat grain treated with the biocontrol agent, the highest drop was observed in the concentrations of alpha/beta-gliadins (10.59%), but grain quality was most affected by an estimated 8% decrease in the content of HMW glutenins.

The protein fractions of the analyzed wheat grain were within the reference ranges (Singh, MacRitchie 2001, Shewry et al. 2002, Konopka et al. 2007). Gliadins had a very high share of gluten proteins, whereas according to published sources, they do not account for more than 60% of gluten proteins in most wheat cultivars. Based on the above protein profile, cv. Bogatka was classified as a bread wheat cultivar with average baking properties.

Table 2

<table>
<thead>
<tr>
<th>Protein fraction</th>
<th>Control</th>
<th>Bacteria</th>
<th>Fungicide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumins+globulins</td>
<td>1.15·10^4a</td>
<td>1.09·10^4ab</td>
<td>1.09·10^4ab</td>
</tr>
<tr>
<td>Gliadins</td>
<td>3.37·10^4b</td>
<td>3.07·10^4a</td>
<td>3.13·10^4ab</td>
</tr>
<tr>
<td>omega</td>
<td>1.89·10^4c</td>
<td>1.69·10^4d</td>
<td>1.74·10^4d</td>
</tr>
<tr>
<td>alfa/beta</td>
<td>1.11·10^4e</td>
<td>1.02·10^4ef</td>
<td>1.03·10^4ef</td>
</tr>
<tr>
<td>glutenins</td>
<td>5.70·10^3a</td>
<td>5.26·10^3a</td>
<td>5.52·10^3a</td>
</tr>
<tr>
<td>HMW</td>
<td>1.26·10^4d</td>
<td>1.20·10^4e</td>
<td>1.25·10^4d</td>
</tr>
</tbody>
</table>

a - f – values assigned the same letter differ not significantly in rows according to SNK-test at p < 0.01.

Higher bacteria and yeast counts on harvested grain of wheat plants protected with a cell suspension of bacteria probably contributed to changes in the grain’s biochemical characteristics. The biocontrol agent reduced the FRQWHQWRIODSKDEHDJOLDGLQV DQG ORZ PROHFXODU ZHLJKW +0: DFRXQWHG IRU 7KH DQDO\\ JHG plant protection agents lowered the content of most protein fractions. 7KH RQO\\ H[FHSWLRQV ZHUHRPHJ D]OLDGLQV DQG ORZ PROHFXODU ZHLJKW /O: glutenins, whose concentrations remained constant. In control grain, the FRQWHQWRIJOXWHQSURWHLQV ZDV KLJKHUWKDQLQIXQJLFLGHWUDWHGJUDLQ and 7.9% higher than in grain treated with the biocontrol agent. In wheat grain treated with the biocontrol agent, the highest drop was observed in the FRQFHQWUDLRQV RIDOSKDEHDJOLDGLQV EXW JUDLQ TXDOLW\\ ZDV PRVW DIIHFWHG E] DQ HVWLPDWG GHFUDHV LQ WKHFQWHQW Ri +0: JOXWHQLQV

The protein fractions of the analyzed wheat grain were within the reference ranges (Singh, MacRitchie 2001, Shewry et al. 2002, Konopka et al. 2007). Gliadins had a very high share of gluten proteins, whereas according to published sources, they do not account for more than 60% of gluten proteins in most wheat cultivars. Based on the above protein profile, cv. Bogatka was classified as a bread wheat cultivar with average baking properties. The biocontrol agent reduced the FRQWHQWRIODSKDEHDJOLDGLQV DQG +0: JOXWHQLQV ZKLFGKHWURQLQHWKHE

Higher bacteria and yeast counts on harvested grain of wheat plants protected with a cell suspension of bacteria probably contributed to changes in the grain’s biochemical characteristics. The biocontrol agent reduced the FRQWHQWRIODSKDEHDJOLDGLQV DQG +0: JOXWHQLQV ZKLFGKHWURQLQHWKHE improving the overall quality of wheat grain (Wieser, Zimmerman 2000, DuPont et al. 2004). There are no studies documenting the effect of bacteria of the genus Spin-gomonas as a biocontrol agent on the biochemical characteristics of wheat grain, including reserve, structural and metabolic proteins. The applied bac-
Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Date of analysis</th>
<th>Yeasts</th>
<th>Bacteria</th>
<th>1XPEHUR</th>
<th>DPHQWRXVIQJ</th>
<th>LFRQORQLHV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>growth on nitrogen-free medium</td>
<td>pseudomonad group</td>
<td>total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A.strictum</td>
<td>A.alternata</td>
</tr>
<tr>
<td>Control</td>
<td>H</td>
<td>c</td>
<td>3.20</td>
<td>A</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>a</td>
<td>3.93</td>
<td>A</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>H</td>
<td>c</td>
<td>3.22</td>
<td>A</td>
<td>2.77</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>a</td>
<td>3.72</td>
<td>A</td>
<td>2.77</td>
<td></td>
</tr>
<tr>
<td>Fungicide</td>
<td>H</td>
<td>b</td>
<td>3.32</td>
<td>3.70</td>
<td>2.31</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>b</td>
<td>3.43</td>
<td>4.09</td>
<td>2.31</td>
<td></td>
</tr>
</tbody>
</table>

2009
- 2.53
- 3.02
- 4.24
- 2.41
- 0.21
- 0.42

2010
- 3.24
- 3.02
- 4.09
- 3.09
- 0.02
- 0.00

2011
- 3.41
- 4.24
- 3.02
- 0.13
- 2.24

H
- 4.02
- 2.77


The abundance and structure of filamentous fungi colonizing grains were determined by the dates of microbiological analyses and the applied control treatments (Table 3). In comparison with control samples, chemical treatment suppressed the growth of filamentous fungi immediately after harvest. The structure of filamentous fungi colonizing winter wheat grains was typical of that environment. In total, 706 colonies were identified with the predominance of species of the genus *Fusarium* (*F. culmorum*, *F. poae*, *F. sporotrichiodes*, *F. avenaceum*), which accounted for 34.1% of all colonies. In 2010-2011, less abundant communities of *Fusarium* fungi were isolated from threshed grain of wheat plants treated with the biocontrol agent and fungicides, especially stored for six months, in comparison with control. A cell suspension of bacteria did not inhibit the growth of yeasts and epiphytic bacteria of the genus *Azotobacter* on grains (Table 3), which improved the health status of wheat plants as yeasts are considered to be potential antagonists of winter wheat pathogens (Zhang et al. 2007).

**CONCLUSIONS**

1. Biological treatment increased winter wheat grains yield and inhibited the growth pathogens of *F. culmorum*, *F. poae*, *F. sporotrichiodes* and *F. avenaceum*.

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