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STUDIES ON CEREAL SOIL-BORNE VIRUSES IN POLAND

Małgorzata Jeżewska, Katarzyna Trzmiel*

Institute of Plant Protection – National Research Institute, Department of Virology and Bacteriology
Węgorka 20, 60-318 Poznań, Poland

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Abstract: Four soil-borne cereal viruses have been identified in Poland, so far: *Soil-borne cereal mosaic virus* (SBCMV), *Wheat spindle streak mosaic virus* (WSSMV), *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV). SBCMV was identified in 1993 as a dangerous pathogen of winter cereals and became the object of special interest. Studies on the virus included its biological and molecular characterization, and investigations of the response of winter wheat and winter triticale cultivars on the SBCMV infection. Results of preliminary experiments aiming at the evaluation of the response of winter barley cultivars on barley yellow mosaic viruses were also presented.

Key words: *Soil-borne cereal mosaic virus*, *Barley yellow mosaic virus*, *Barley mild mosaic virus*, resistant cultivars

INTRODUCTION

Cereal viruses transmitted by the plasmodiophoric soil organism, *Polymyxa graminis* Led., called "soil-borne" viruses, are dangerous pathogens. The longevity of virus particles in spores of their vector enables the persistence of the inoculum in the soil for many years (Adams *et al.* 1988, 1993). This is particularly harmful in view of the limited possibilities of crop rotation in Poland with a definite predominance of cereals.

The first soil-borne cereal virus identified in Poland was the *Soil-borne cereal mosaic virus*, SBCMV, (Jeżewska 1994). The virus was initially identified as a strain of *Soil-borne wheat mosaic virus* (SBWMV) but since 2000 it was classified as SBCMV (Koenig and Huth 2000a, b). According to literature data, SBWMV is considered a very dangerous pathogen (Canova and Quaglia 1960; Vallega *et al.* 1999a, b; Clover *et al.* 2001; Budge *et al.* 2002). For this reason, the virus became the object of our studies. We aimed at characterizing the biological and molecular features of the virus. We also aimed at examining the response of chosen winter wheat and winter triticale cultivars to the infection. In the epidemiology of SBCMV, the seed transmission capacity of the virus may play an important role (Garbaczewska *et al.* 1997; Jeżewska 2006; Budge *et al.* 2008).

In the last years, three other soil-borne viruses were found: *Wheat spindle streak mosaic virus* (WSSMV), *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) (Jeżewska and Trzmiel 2007; Jeżewska and Trzmiel 2009a, b). Surprisingly, WSSMV was isolated from triticale plants showing symptoms of mild leaf mosaic. In the following years, the virus was detected only sporadically in wheat plants, usually accompanying SBCMV. The causal agents of barley yellow mosaic; BaYMV and BaMMV, were isolated from severely diseased barley plants. Taking into account the potential risk involved in the occurrence of these pathogens (Huth 1989; Plumb *et al.* 1986), investigations were undertaken in order to determine their distribution in the country. Assays were also done to evaluate the response of some winter barley cultivars to BaYMV and BaMMV.

The objective of this paper was to present the results of our investigations on soil-borne cereal viruses in Poland.

MATERIALS AND METHODS

Plant samples with disease symptoms suggesting infection with viruses were collected.

Diagnostics were initially performed by ELISA tests (Clark and Adams 1977). Commercial kits for TAS-ELISA and DAS-ELISA produced by Loewe (Germany) and Neogen (Great Britain) were also used.

SBCMV isolates

Five SBCMV isolates, originating from different locations in Poland, were taken for investigation: SBCMV-Cer (Cerekwica), SBCMV-Ch (Choryń), SBCMV-Chd (Chude), SBCMV-St (Strzelce) and SBCMV-Żab (Żabienko).

The viruses were easily propagated in a glasshouse and climatic chamber, by soil-transmission experiments.

RNA isolation

RNA was isolated from fresh infected plant leaves. Total RNA extraction from about 100 mg of plant material was carried out with the use of the RNeasy Mini Kit (Qiagen), according to the procedure supplied by the producer. The RNA was eluted with 40 µl RNase-free water.

*Corresponding address:
m.jezewska@iorpib.poznan.pl

RT-PCR

The one step RT-PCR kit (Qiagen) was used to obtain and subsequently amplify cDNA fragments. RT-PCR was carried out using 1 µl template RNA, 1 µl forward and 1 µl reverse primers (10 µM), 2 µl 5×Qiagen OneStep RT-PCR Buffer, 0.4 µl dNTP Mix (10 mM), 0.4 µl Qiagen OneStep RT-PCR Enzyme Mix, 10 units of RiboLock RNase inhibitor (Fermentas) in a total of 10 µl volume. The reactions were performed using T-personal thermocycler (Biometra) as follows: first, a reverse transcription for 30 min at 50°C and an initial PCR activation step for 15 min at 95°C were done, then 35 cycles including denaturation for 1 min at 94°C, annealing for 1 min – depending on primer temperature and elongation for 1 min at 72°C, were carried out. A final elongation was completed at 72°C for 10 minutes. The annealing temperature was optimized by testing a gradient of temperatures on control samples, in T- Professional thermocycler (Biometra). All primers and their annealing temperatures were listed on table 1. SBCMCP-F and SBCMCP-R primers were designated to RNA2 complete sequence of French isolate of SBCMV (Accession No. AJ132577) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi) (Rosen and Skaletski 2000). The primer pairs amplified coat protein fragment of SBCMV.

DNA cloning and sequencing

RT-PCR products were separated electrophoretically in 1% agarose gel and visualised in UV after staining with ethidium bromide. Reaction products of the expected sizes: 978 bp with SBCM-F/SBCMV-R and 596 bp with SBCMCP-F/SBCMCP-R primer pairs were excised from the gel and then eluted using QIAEX II Gel Extraction Kit (Qiagen). The PCR products were ligated into the pGEM-T Easy vector (Promega), and transformed into *E. coli* DH5α cells (Invitrogen). DNA of positively verified plasmids were isolated after overnight culturing using QIAprep Spin Miniprep Kit (Qiagen). Two independently amplified and cloned PCR products were sequenced in both directions using M13-F and M13-R primers and (DTCS) Quick Start Kit (Beckman Coulter). All steps were performed according the producers instructions. Obtained nucleotide sequences were analysed by BlastN program and manually edited in GeneDoc.

Phylogenetic analysis

Multiple alignment was performed using ClustalW software. Other viral sequences used for a comparison were retrieved from the GenBank database (Table 2). MEGA 3.1 software with neighbour – joining method (NJ), was used for phylogenetic analyses of the obtained the nucleotide and deduced amino acid sequences (Kumar *et al*. 2004). The reliability of the NJ trees was assessed using 1 000 bootstrap replicates.

Evaluation of the response of winter wheat and winter triticale cultivars on SBCMV infection

Two kinds of experiments were performed; experiments in a glasshouse, and a field trial experiment.

The glasshouse experiments were conducted in early spring, with temperatures not exceeding 20°C. Plants of each cultivar were placed in boxes with infectious soil. Evaluation of results was done after 7–8 weeks using TAS-ELISA tests. Each box contained 20 plants. For each cultivar 40–60 plant samples were analyzed, depending on the number of repetitions of the experiment. Two SBCMV isolates were used in the experiment: SBCMV-Ch and SBCMV-St.

The field trial was localized in Choryń (the Wielkopolska region), on a field previously confirmed to be infested with natural SBCMV bearing vector. Twelve winter wheat cultivars: Alkazar, Bogatka, Boomer, Brillant, Figura, Finezja, Legenda, Muszelka, Naridana, Ostroga, Smuga and Tonacja were evaluated. The cultivars were grown in plots of 2 m^2, each in 5 repetitions, sown 20 September 2007. Observation of symptoms and collection of samples to be tested for SBCMV infection were done 11 March 2008. Ten plants were taken from each plot (50 per cultivar) for the TAS-ELISA test.

Table 1. List of primers used for RT-PCR

Primer name	Annealing temperature	Primer sequence 5'–3'	References
sb11 (forward)	50°C	TGG GCC GGA TAA CCC T	Koenig and Huth 2000a
sb55 (reverse)	50°C	GAG AAT CGG AAA AAT CAC TAT GAT	Koenig and Huth 2000a
sb20 (forward)	50°C	AGT GGG AAG GTA CGA GTT GA	Koenig and Huth 2000
sb40 (reverse)	50°C	CCA CGC TTT CCC ATT CAT CAA ATT G	Koenig and Huth 2000
SBCMV-F	56°C	ACT TAC CCA TTT AGG TGT AA	Fomitcheva *et al*. 2009
SBCMV-R	56°C	TTA TAA TCA CGC AAG TAC CT	Fomitcheva *et al*. 2009
SBCMCP-F	64°C	AAT CGA AAG TGG TTG TGC AGT	own-unpublished
SBCMCP-R	64°C	AAT GCG TGC CCT CAA CTT T	own-unpublished
BaYMV-F	58°C	AAA GCC TGG ACT GAT GCT GT	Vaïanopoulos *et al*. 2003
BaYMV-R	58°C	GTG GGA CGA AGA AAT CGA AA	Vaïanopoulos *et al*. 2003

Table 2. SBCMV and SBWMV nucleotide sequences used for comparison and phylogenetic analysis

Virus species	Isolate	Country	GenBank accesion number
SBCMV	C	Germany	AF146281
	G	Germany	AF146282
	O	Germany	AF146283
	Ozzano	Italy	AJ252152
	Chambon-sur-Cisse	France	FN298366
	Cezanne	France	FN298365
	Flavion	Belgium	FN298364
	France	France	AJ132577
SBWMV	Wiltshire	UK	AJ298069
	Kent	UK	AJ298070
	JT	Japan	AB033690

Evaluation of the response of winter barley cultivars to barley yellow mosaic viruses (BaYMV and BaMMV) infection

Preliminary experiments were conducted in glasshouse conditions and in a growth chamber. Two methods of inoculation were compared: mechanical, and by infested soil. Mechanical inoculation was performed according to the procedure described by Kuntze *et al.* (2000). In soil experiments, plants of each cultivar were placed in boxes with infested soil (20 plants per box). The test plants were cultivated in the growth chamber under precisely controlled temperature conditions: (10°C during night, 12°C during a 12-h day). Evaluation of the results for BaYMV and BaMMV infection, using the DAS-ELISA test, was carried out after 4 weeks (in the case of mechanical inoculation) and after 6–7 weeks (in the case of soil inoculation).

RESULTS AND DISCUSSION

Occurrence of SBCMV, BaYMV and BaMMV

Distribution of SBCMV in Poland in 2010 was presented in figure 1. In the course of routine SBCMV monitoring it was demonstrated that its expansion was rather slow. However, the virus was actually detected in 8 voivodeships, covering an area of more than half the country. Disease symptoms of SBCMV infection included leaf mosaic, leaf yellowing, weaken plant growth, and occasionally, stunting.

Barley yellow mosaic viruses have been monitored since 2008, when they were detected in the Lower Silesia region (Jeżewska and Trzmiel 2009a, b). In 2010 the viruses already occurred in 8 voivodeships. Almost all the voivodeships were those where winter barley was grown, except for the Opole region, as shown in figure 2. In infected plants the viruses caused mild mosaic, leaf yellowing and decreased growth.

Both in the case of SBCMV and barley yellow mosaic viruses in the fields, irregular yellow patches could be seen associated with infections.

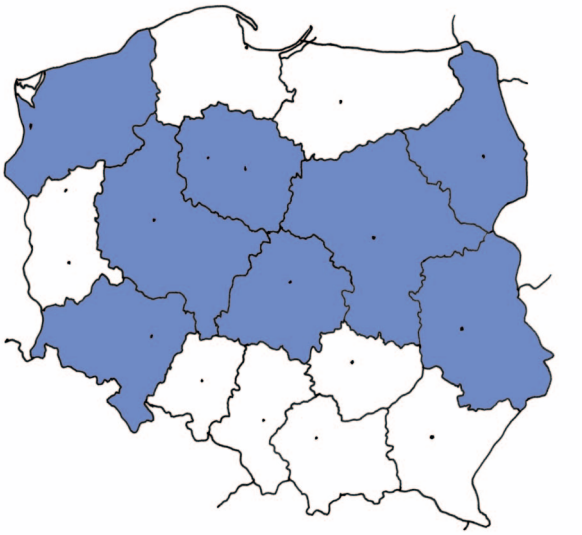

Fig. 1. Occurrence of *Soil-borne cereal mosaic virus* in Poland in 2010

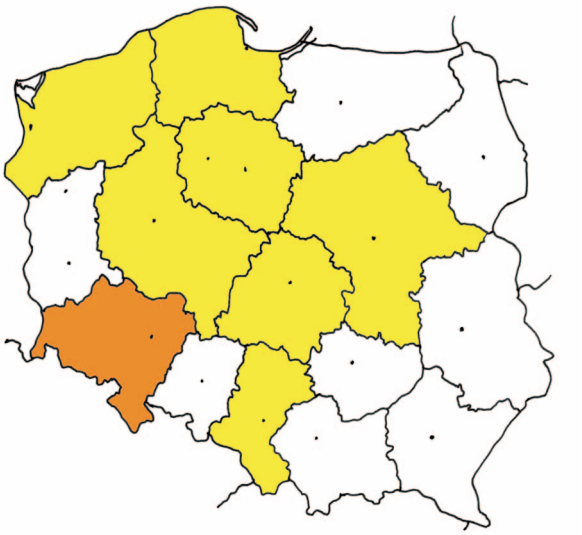

Fig. 2. Occurrence of *Barley yellow mosaic virus* and *Barley mild mosaic virus* in Poland in 2010

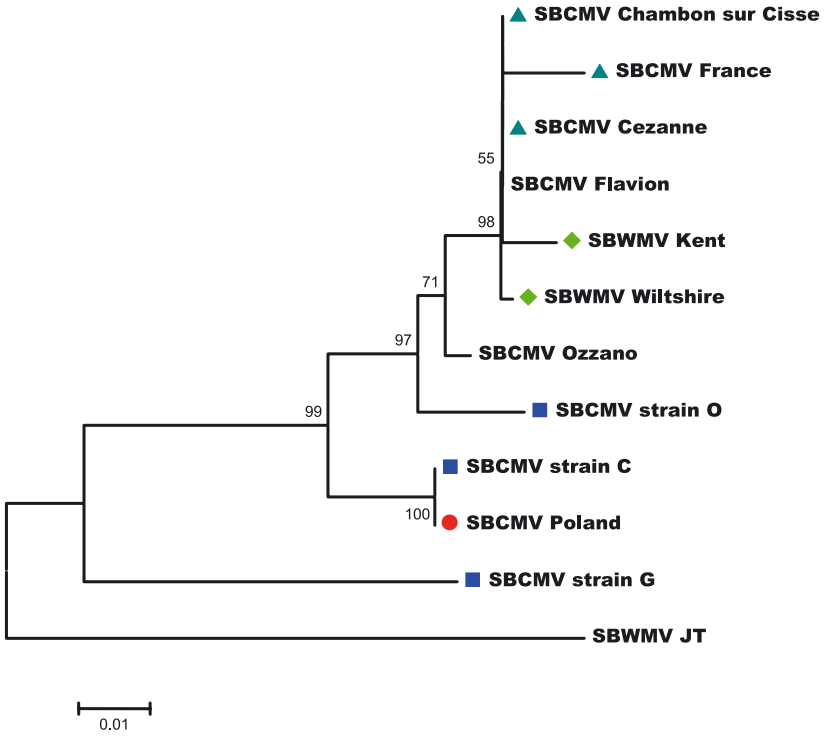

Fig. 3. Dendrogram of SBCMV isolates based on nucleotide sequence of the 1134 bp fragment of RNA 2 (276-1410 nt). Phylogenetic tree was constructed using the neighbor joining method of MEGA 3.1.

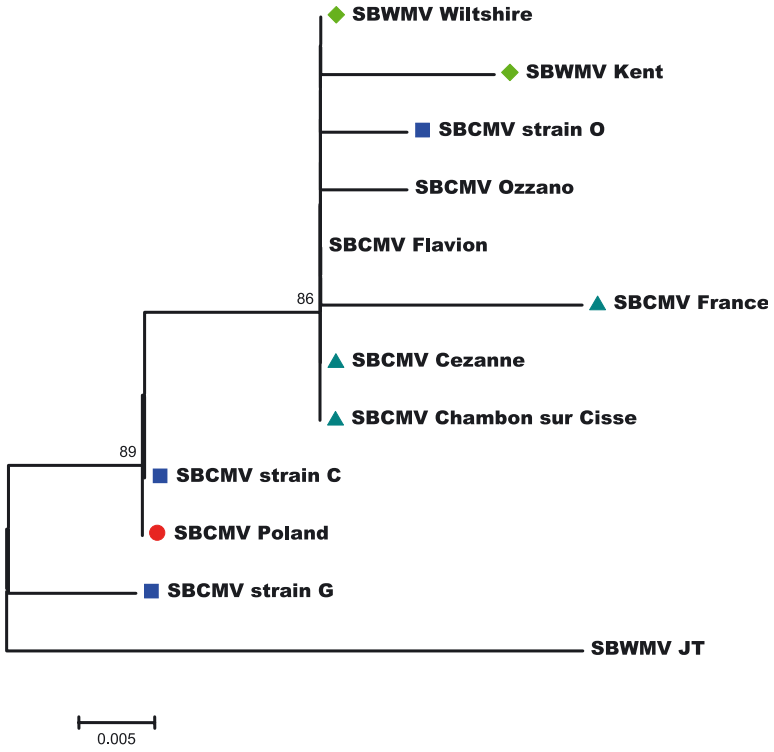

Fig. 4. Dendrogram of SBCMV isolates based on deduced 378 aa long amino acid sequences of RNA 2 (276-1410 nt). Phylogenetic tree was constructed using the neighbor joining method of MEGA 3.1.

Molecular characterization and comparison of Polish isolates of SBCMV

1 134 bp long sequences of Polish strains of SBCMV were determined after joining sequences obtained from two PCR products. This fragment corresponded to nucleotides 276 to 1 410 bp of RNA2 of SCMV genome strain C (GenBank Accession No. AF146281). The determined region consisted of the CP and a part of the CP-RT genes. It showed the highest nucleotide sequence identities (99%) with German SBCMV isolate strain "C" (AF146281). All Polish isolates were homologous. Polish isolates shared high identities which ranged from 97 to 98%. The phylogenetic trees were constructed based on 1134 bp long nucleotide and deduced amino acid sequence alignments. Topologies of both phylogenetic trees were similar. The results revealed the Polish strain of SBCMV to be most closely related to the German SBCMV isolates.

Reaction of winter wheat and winter triticale cultivars to SBCMV infection

Evaluation of the response of winter wheat and winter triticale cultivars on SBCMV infection was carried out both in a glasshouse and in the field.

Reactions to SBCMV of 16 winter wheat and 2 winter triticale cultivars in the glasshouse experiments were evaluated on the basis of symptom expression and TAS-ELISA results (Table 3). The results are summarized as there was no significant differences between the rate of infections for the cultivars depending on the isolate of SBCMV used in the experiment. There was also no difference concerning visible symptoms of the infection. In every case the only disease symptom observed was attenuated plant growth accompanied by mild leaf yellowing. The mild character of the infections was reflected also in the low level of OD values, in plants found infected in the

Table 3. Reaction of winter wheat and winter triticale cultivars on SBCMV infection in glasshouse experiments

Cultivar	Number of plants tested	Percentage of SBCMV infected plants	Range of OD values in TAS-ELISA*
Wheat			
Alkazar	40	27.5	0.05–0.1
Batuta	40	12.5	0.03–0.05
Bogatka	60	6.7	0.03–0.08
Boomer	40	5.0	0.05–0.1
Brillant	40	5.0	0.04–0.15
Figura	60	1.7	0.05
Finezja	40	7.5	0.04–0.07
Legenda	60	6.7	0.05–0.08
Muszelka	60	5.0	0.04–0.06
Naridana	40	15.0	0.03–0.09
Ostoja	40	0	< 0.01
Ostroga	40	2.5	0.05
Smuga	40	0	< 0.01
Tonacja	60	8.3	0.04–0.35
Turkis	40	2.5	0.03
Turnia	40	7.5	0.03–0.11
Triticale			
Grenado	60	16.7	0.03–0.1
Moderato	60	10.0	0.04–0.22

* OD for healthy plants < 0.01

Table 4. Reaction of winter wheat cultivars on SBCMV infection in a field experiment, Choryń 2007/2008 (50 plant samples were tested using TAS-ELISA, from each cultivar)

Cultivar	Number of plants found infected with SBCMV	Range of OD values in TAS-ELISA*	Yield [dt/ha]
Alkazar	1	0.04	69.6
Bogatka	7	0.03–0.11	80.3
Boomer	8	0.03–0.09	72.9
Brillant	18	0.04–0.09	75.0
Figura	5	0.03–0.09	76.9
Finezja	3	0.03–0.08	82.9
Legenda	1	0.05	82.8
Muszelka	9	0.03–0.13	91.8
Naridana	8	0.04–0.09	85.6
Ostroga	3	0.04–0.06	86.4
Smuga	0	0	79.0
Tonacja	2	0.05–0.11	73.8

* OD for healthy plants < 0.01

Table 5. Reaction of winter barley cultivars on BaYMV and BaMMV infection in two experiments (in each experiment 20–32 plant samples were tested in TAS-ELISA per each cultivar)

Cultivar	Experiment 1: soil infection in glasshouse					Experiment 2: mechanical inoculation in growth chamber				
	percentage of plants			range of OD values in DAS-ELISA		percentage of plants			range of OD values in DAS-ELISA	
	infected with		healthy	BaYMV	BaMMV	infected with		healthy	BaYMV	BaMMV
	BaYMV	BaMMV				BaYMV	BaMMV			
Amarena	18.8	21.9	75.0	0.04–0.22	0.08–0.19	–	–	–	–	–
Bartosz	0	0	100	< 0.01	< 0.01	0	14.3	85.7	< 0.01	0.03–0.08
Bażant	0	0	100	< 0.01	< 0.01	9.5	33.3	66.7	0.04–0.09	0.03
Bursztyn	0	0	100	< 0.01	< 0.01	–	–	–	–	–
Epoque	25	0	75	0.03–0.05	< 0.01	4.8	33.3	61.9	0.06	0.03–0.05
Fridericus	12.5	0	87.5	0.03–0.19	< 0.01	23.8	42.9	47.6	0.03–0.04	0.03–0.06
Gil	31.1	25.0	50.0	0.03–0.20	0.03–0.05	19.0	42.9	52.4	0.03–0.05	0.03–0.07
Horus	3.1	0	96.9	0.17	< 0.01	–	–	–	–	–
Karakan	6.2	9.3	87.5	0.04–0.08	0.03–0.05	28.6	14.3	66.7	0.03–0.06	0.03–0.05
Lomerit	13.2	0	86.8	0.08–0.13	< 0.01	0	0	100	< 0.01	< 0.01
Maybrit	6.0	0	94.0	0.03–0.18	< 0.01	9.5	0	90.5	0.03–0.05	< 0.01
Merlot	28.9	0	71.1	0.04–0.21	< 0.01	9.5	0	90.5	0.03–0.06	< 0.01
Nickela	0	15.5	85.0	< 0.01	0.03–0.04	0	6.3	93.7	< 0.01	0.03–0.04
Rosita	0	0	100	< 0.01	< 0.01	4.7	0	95.3	0.11	< 0.01
Scarpia	9.4	3.1	87.5	0.10–0.25	0.15	0	0	100	< 0.01	< 0.01
Traminer	9.4	0	90.6	0.06–0.11	< 0.01	10.0	5.0	90.0	0.05–0.16	0.05
Vanessa	25.0	8.3	70.8	0.03–0.28	0.03–0.04	–	–	–	–	–
Wintmalt	6.2	15.6	78.1	0.03–0.21	0.03–0.20	0	0	100	< 0.01	< 0.01

* OD for healthy plants < 0.01

ELISA test. Surprisingly, the rate of infections detected in triticale plants was limited. These findings are in contrast to previous observations during routine field inspection monitoring where SBCMV infections in triticale were encountered more often than in wheat.

Table 4 demonstrates results of a field trial carried out in Choryń in the years 2007–2008. The data confirmed that Polish isolate SBCMV-Ch proved not to be aggressive and did not impact seriously on the yield of winter wheat cultivars in the experiment. These observations remained in contrast with the data provided by Turkish, British and Italian virologists (Vallega *et al.* 1997; Rubies-Autonell *et al.* 2000; Budge *et al*. 2002; Altay and Bolat 2004). The above authors reported a broad scale of winter wheat cultivar reactions to SBCMV infection pressure in field conditions. The reactions included examples of serious susceptibility resulting in important crop yield decrease.

In cultivars: Bogatka, Boomer, Figura, Finezja, Legenda, Naridana, Ostroga and Tonacja, an approximately similar, *i.e.* limited rate of SBCMV infection was recorded both in the glasshouse and in the field experiments. However, there were also examples of discrepancy in the results of the two kinds of experiments. Two cultivars, Brillant and Muszelka, which appeared to be resistant in the glasshouse experiment, showed high levels of infection in the field trial. The opposite situation was observed in the case of cv. Alkazar. Smuga seemed to be the most promising cultivar, as no SBCMV infection was detected in both experiments.

Investigations on the reaction of winter wheat cultivar to the infection with SBCMV led us to the conclusion, that the risk of important crop losses caused by the virus is actually moderate, for the mild character of Polish isolates. We were also led to conclude, that perhaps there is a tolerance of Polish plant materials.

Reaction of winter barley cultivars to BaYMV and BaMMV infection

Results of two experiments, evaluating the reaction of 18 winter barley cultivars to the barley yellow mosaic viruses infection, are summarized in table 5 (as the viruses were detected in mixed infection or separately, the percentages of healthy plants were also given). According to the literature, three methods of screening for resistance of barley to BaYMV have been accepted as necessary: pretest, main test and field test (Proeseler *et al.* 1991). In our investigation, field testing was lacking to conclude reliably about the resistance. Nevertheless, these data seem to initially point out cultivars that appear susceptible. Some of the results obtained in the tests were supported by data from field monitoring (Vanessa, Wintmalt). These cultivars should not be grown in areas infested with barley yellow mosaic viruses. In the case of other cultivars, field tests are necessary to evaluate their response to the pathogens.

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POLISH SUMMARY

BADANIA ODGLEBOWYCH WIRUSÓW ZBÓŻ W POLSCE

Wirusy zbóż określane jako „odglebowe" (ang. „soil-borne") są przenoszone przez pierwotniaka glebowego, *Polymyxa graminis* Led. W Polsce do 2010 roku zidentyfikowano następujące odglebowe wirusy zbóż: odglebowej mozaiki zbóż (*Soil-borne cereal mosaic virus*, SBCMV), wrzecionowato-smugowatej mozaiki pszenicy (*Wheat spindle streak mosaic virus*, WSSMV), żółtej mozaiki jęczmienia (*Barley yellow mosaic virus*, BaYMV) oraz łagodnej mozaiki jęczmienia (*Barley mild mosaic virus*, BaMMV).

Celem badań wirusów odglebowych było: określenie zasięgu występowania SBCMV, BaYMV i BMMV, charakterystyka biologiczna i molekularna polskich izolatów SBCMV oraz poszukiwanie odporności u odmian roślin gospodarzy.

Głównym obiektem badań był SBCMV. Przebadano 5 izolatów tego wirusa, pochodzących z różnych rejonów Polski. Nie stwierdzono zróżnicowania właściwości bio-

logicznych badanych izolatów. W badaniach molekularnych, przy zastosowaniu starterów literaturowych oraz własnych sklonowano i poddano sekwencjonowaniu fragment RNA2 wirusa, o długości 1 138 nt (od 276 do 1 410 nt), obejmujący gen białka kapsydu oraz, częściowo, białka CP-RT. Porównanie sekwencji nukleotydowych wykazało 99% podobieństwa polskiego izolatu do niemieckiego izolatu AF 146281. Porównanie sekwencji nukleotydowych polskich izolatów SBCMV również potwierdziło ich bardzo wysoki procent identyczności (97–98). Ważnym osiągnięciem było odkrycie zdolności SBCMV do przenoszenia się przez nasiona żyta. Możliwość przenoszenia przez nasiona ma poważne konsekwencje epidemiologiczne i stanowi stosunkowo łatwy sposób przemieszczania się wirusa na dalekie odległości. Zebrane dane dotyczące reakcji odmian pszenicy i pszenżyta stanowią podstawę opracowania zaleceń w zakresie ochrony upraw przed wirusami odglebowymi.

Badania w zakresie wirusów wywołujących objawy żółtej mozaiki jęczmienia, BaYMV i BaMMV, koncentrowały się na rozpoznaniu rejonizacji ich występowania oraz na wstępnej ocenie reakcji odmian jęczmienia ozimego na porażenie w doświadczeniach szklarniowych i w komorze klimatycznej. Uzyskane wyniki pozwoliły na wyłonienie odmian zdecydowanie podatnych na porażenie przez wirusy, których należy unikać w rejonach zagrożonych występowaniem wirusów żółtej mozaiki jęczmienia.