EVALUATION OF THE AUTUMN INFECTION OF WINTER BARLEY WITH BARLEY YELLOW DWARF VIRUSES TRANSMITTED BY ANHOLOCYCLIC FORMS OF BIRD CHERRY-OAT APHID *RHOPALOSIPHUM PADI* L. IN POLAND

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Abstract: Research was carried out to determine the extent of anholocyclic forms of bird cherry-oat aphid, *Rhopalosiphum padi* on winter barley, and to estimate the level of infection of winter barley crops with Barley yellow dwarf (BYD) viruses. Observations were made in 12 Polish regions. Each region is made up of four distinct locations, with different temperatures. The 12 observed regions were: Lubuskie, Dolnośląskie, Opolskie, Śląskie, Małopolskie, Podkarpackie, Wielkopolskie, Łódzkie, Mazowieckie, Lubelskie, Warmińsko-Mazurskie and Podlaskie. The research was carried out during the period of colonization of plants by aphids. Anholocyclic forms of *R. padi* were found on winter barley crops in all regions, with the exception of the Podlaskie area. Samples of plants were collected and tested for virus occurrence by ELISA. In 2007, the detection of BYD viruses in aphids feeding on winter barley was performed using the PCR technique. Virus diagnostics revealed the prevalence of *Barley yellow dwarf virus-PAV* (BYDV-PAV) over *Barley yellow dwarf virus-MAV* (BYDV-MAV), in 2006 and 2007. Aphid vectors of BYD viruses were the most numerous in all the locations of the Opolskie region.

Key words: aphids, *Rhopalosiphum padi*, barley yellow dwarf viruses

INTRODUCTION

Recently in Poland an increase in the number of aphids infesting cereals has been observed. The diversity of species and forms of aphids, the ability to migrate, and the quick adaptation-reaction to any environmental changes, mean these insects are becoming increasingly important in plant protection (Leszczyński 1985; Ruszkowska 1988, 1990a, b 2002; Eastop 1995; Dixon 1998; Giebel *et al*. 1998; Martinez-Torrez *et al*. 1997; Mrówczyński *et al*. 2004). One of the factors causing new potential threats to cereals is the higher temperature in some regions of Poland, which switch the holo- into anholo- life cycle. High temperatures during spring and summer are necessary for the development of anholocyclic forms of cereal aphids in the natural environment. Temperature monitoring is a key element in the projection of these important developmental changes of aphids. A few days of temperature ≥ 25°C will induce the development of these forms in the spring and summer. Temperature ≥ 25°C is the first sign of the possibility of the presence of cereal virus infection (Ruszkowska 1998, 2002, 2006, 2007a, b; Ruszkowska and Strażyński 2007). The dominating species among cereal aphids in Poland is the bird cherry-oat aphid (*Rhopalosiphum padi* L.) and anholocyclic forms of this species are the most important vectors of BYDV (Jeżewska *et al*. 2010).

Aphid vectors of plant viruses may play an important role in the epidemiology of certain diseases, such as Barley yellow dwarf (BYD). Symptoms of BYD are caused by several small spherical viruses transmitted exclusively by aphids in a persistent manner (Plumb 1992; Power *et al*. 1991). The main causal agents of BYD in Poland are: *Barley yellow dwarf virus-MAV* (BYDV-MAV), *Barley yellow dwarf virus-PAV* (BYDV-PAV and *Cereal yellow dwarf virus-RPV* (CYDV-RPV). BYD previously did not have a significant economic impact on the yield of cereals in Poland. Nowadays however, BYD is considered to be a serious potential danger particularly for winter barley crops. The economic importance of BYD associated with the increase of anholocyclic aphid populations was reported (Badillo-Vargas and Gildow 2004; Lowles *et al*. 1999).

The common presence of *R. padi* on winter cereals enhances the potential danger of early infections with BYD viruses (Strażyński 2010). The increase of BYD occurrence in recent years was recorded (Jeżewska 2003; Jeżewska *et al*. 2010).

Therefore, in the years 2006–2008, studies were undertaken in order to recognize the structure of the population of *R. padi*, depending on the temperatures in different regions of the country. An additional objective was to check the impact of the occurrence of anholocyclic forms

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of *R. padi* on the autumnal infections of winter barley plants with BYD viruses.

The first aim of our study was to provide an update on the proportions of anholocyclic forms of *R. padi* conditioned by proper temperature. The second aim of our study was to determine the dynamics of changes in the diversity of forms and morphs.

MATERIALS AND METHODS

Entomological research

The study was conducted in 2006–2008, to determine the territorial range of anholocyclic forms of *R. padi*. Observations were made in twelve Polish regions, all with different temperatures: Dolnośląskie, Lubelskie, Lubuskie, Łódzkie, Małopolskie, Mazowieckie, Opolskie, Podkarpackie, Podlaskie, Śląskie, Warmińsko-Mazurskie and Wielkopolskie.

Studies for the presence of anholocyclic forms were conducted during the summer (harvest maturity) and autumn (after emergence of cereals). These forms infesting mature cereals were identified on the basis of the mode of plant colonization. Anholocyclic *R. padi* preferred to inhabit lower parts of stalks, just off the ground, while holocyclic forms fed on the upper parts of the plants.

Observations were carried out on 4 fields in different locations following the diagonal, each time on 100 plants selected at random. Similar observations were carried out in autumn from the emergence to the onset of –6°C temperature, which is a critical temperature for aphid development. The specificity of spatial colonization of cereals within the entire crop had to be taken into account in the field observations (Dean 1970).

Throughout the period of the research, the course of the temperatures in all locations was monitored using a web application Weatherscope (http//weatherscope.com). The collected data was analyzed in terms of sums of daily average temperature; the number of days per year with average daily temperature ≥ 25°C.

Virus diagnostics

Materials

The detection of BYD viruses was carried out in plants and aphids.

Plants

Plant samples were mainly winter barley plants infested with aphids, collected in fields during autumn inspections in different regions of Poland. Occasionally, samples of wild grasses were collected for tests, too. The collection of samples was performed as follows:

– in each region of inspection five locations were chosen, distanced at least 20 km from each other,
– in each field five samples were taken and each sample consisted of ten leaves,
– wild grass species for analyses included *Dactylis glomerata*, *Poa annua*, *Setaria viridis*) and *Elymus regens*.

Aphids

R. padi aphids tested for the presence of BYD viruses were collected on winter barley fields in autumn.

Methods

Preparation of plant samples for virus diagnostics

Plant samples were placed in climatic chambers at 20°C, with 70% humidity and 12 hr daylight.

Assessment of the infectivity of aphids as vectors of BYD viruses

Aphids were reared in the conditions described above, to obtain a higher virus concentration and an increase in vector population. At the same time, in the same conditions, the seedlings of winter barley cv. Tiffany were inhabited by part of the same aphid population which migrated in a natural way from field plants. The plants were sown into 8 pots of 17 cm diameter, 20 seeds in each pot. After three weeks, aphids and plants were taken for virus diagnostics.

The detection of BYD viruses

For routine diagnostics of BYD viruses in plants, the ELISA test was used (Clark and Adams 1977). Commercial immunoglobulins and conjugates were provided by Loewe (Germany) and Adgen (Neogen) (UK). In the years 2006 and 2007, the assays were performed for only two BYD causal agents which were: BYDV-MAV and BYDV-PAV, and in 2008 CYDV-RPV was also included.

The molecular method of reverse transcription – polymerase chain reaction (RT-PCR) was also applied as a reference diagnostic test for the detection of BYD viruses in plants and aphids.

The detection of the viruses in aphids was carried out: directly using RT-PCR, and indirectly by feeding the field aphids on healthy plants and testing the plants after the period of incubation (minimum 3 weeks), by ELISA.

Molecular detection of BYD viruses

Total RNA isolation from about 120 mg of plant material or about 10 aphids was carried out with the use of the RNeasy Mini Kit (Qiagen), according to the procedure description supplied by the producer. The RNA was eluted with 40 µl RNase-free water. One- step RT-PCR kit (Qiagen, Germany), with literature primers Lu 1 and Lu 4 (Robertson *et al.*, 1991) and with M3 and M4 (Biskiens *et al*. 2004), was used to confirm the presence of BYDV-PAV and BYDV-MAV, respectively. Lu1/Lu4 and M3/M4 primer pairs amplified 531 bp and 650 bp fragment of coat protein of the studied viruses. The primers Act1/Act2 amplified 320 bp fragment of a conserved region of insect actin (Canning *et al*. 1996), were applied as an internal control for virus detection in aphids. RT-PCR was carried out in total 10 µl volume containing: 1 µl template RNA, 1 µl forward and 1 µl reverse primers (10 µM), 2 µl 5 x 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). BYDV-MAV detection included: a reverse transcription for 30 min at 50°C and an initial PCR activation step for

15 min at 95°C and 2 min at 94°C, followed by 35 cycles including denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C and elongation for 90 sec at 72°C. A final elongation was completed at 72°C for 10 minutes. BYDV-PAV detection was performed as follows: after a reverse transcription for 30 min at 50°C and an initial PCR activation step for 15 min at 95°C, 35 cycles including denaturation for 45 sec at 94°C, annealing for 1 min at 59°C and elongation for 1 min at 72°C, and a final elongation at 72°C for 10 minutes, were carried out.

RT-PCR products were separated electrophoretically in 1% agarose gel and visualised in UV after staining with ethidium bromide.

RESULTS

Aphids on winter cereals in the field observations

The numbers of anholocyclic forms registered on winter barley in the summer is presented in table 1. In most locations, the aphids were found in clusters at the edges of crops, very rarely in the depth of field. The greatest abundance of anholocyclic *R. padi* were found in July 2006, in the regions of Opolskie and Wielkopolska. The least numerous forms of these occurred in 2006–2007, on crops in the Warmińsko-Mazurskie region.

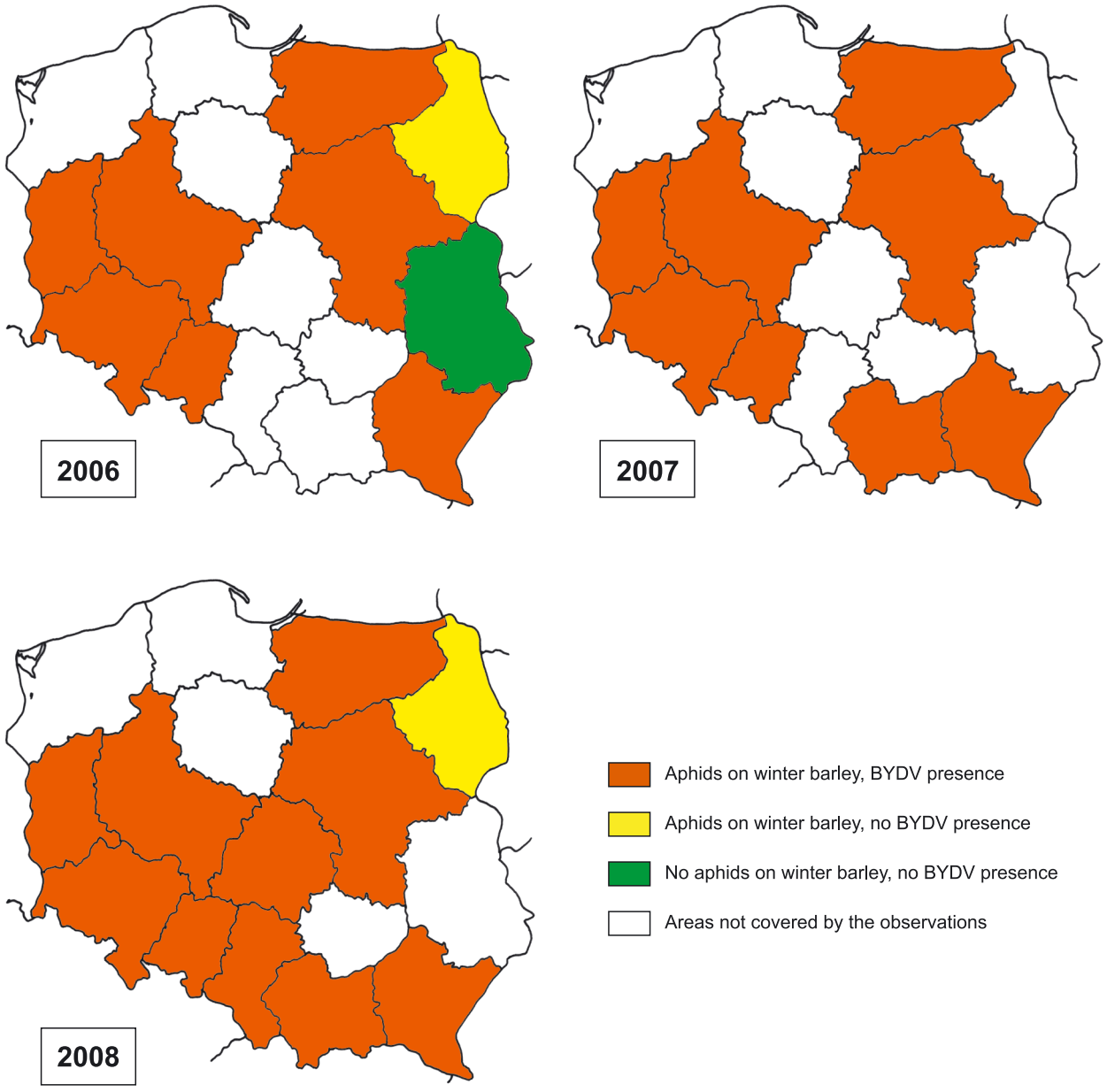

Fig. 1. *R. padi* on winter barley in autumn and BYDV presence in 2006–2008, in Poland

In 2006–2008, in climatically diverse regions of Poland, the autumnal winter cereal crops were observed for the presence of aphids feeding on plants, and the degree of infection by BYD viruses. The territorial expansion of anholocyclic *R. padi* and BYD is shown in figure 1.

Table 1. Anholocyclic forms of *R. padi* on winter barley at maturity stage in the years 2006–2008, in the studied regions

Term of observation	Region	Number of anholocyclic forms on mature plants*		
		2006	2007	2008
July	Wielkopolskie	68	11	48
July	Opolskie	70	26	42
August	Wielkopolskie	38	21	34
August	Warmińsko-Mazurskie	23	12	46
August	Podkarpackie	42	16	32

*average from 100 plants from 4 fields

In 2006, the observations were performed in nine regions: Lubuskie, Wielkopolskie, Dolnośląskie, Opolskie, Podkarpackie, Lubelskie, Mazowieckie, Podlaskie and Warmińsko-Mazurskie. The occurrence of aphids in winter crops and the simultaneous infection of BYD viruses was found in 7 of them: Lubuskie, Wielkopolskie, Dolnośląskie, Opolskie, Warmińsko-Mazurskie, Mazowieckie and Podkarpackie. In the Lubelskie region, aphids were observed on winter crops, but the plants were not infected by viruses. In 2006, in the Podlaskie region, there were no aphids colonizing winter crops and no infected plants.

In 2007, the observations were carried out in the regions of Lubuskie, Wielkopolskie, Dolnośląskie, Opolskie, Małopolskie, Podkarpackie, Mazowieckie and Warmińsko-Mazurskie. All aphids found on winter barley were vectors of BYDV in every region covered by our observations.

In 2008, observations were made in the same areas as in 2007, but the observations were extended into the following regions: Łódzkie, Małopolskie and Podlaskie. In the Podlaskie region, as in 2006, there were no aphids on winter crops and no infected plants. In the remaining regions, aphids settling winter cereals as well as infection of plants were registered.

Temperature in the studied regions inducing the development of anholocyclic forms of aphids

Table 2 shows the number of days with the mean daily temperature which induces the development of anholocyclic forms of *R. padi*, ≥ 25°C.

Table 2. Temperature switching development of the anholocyclic *R. padi* in the years 2006–2008, in selected regions of Poland

Region	2006		2007		2008	
	number of days with temperature ≥ 25°C	date of first day with an average daily ≥ 25°C	number of days with temperature ≥ 25°C	date of first day with an average daily ≥ 25°C	number of days with temperature ≥ 25°C	date of first day with an average daily ≥ 25°C
Wielkopolskie	17	7.07	6	15.07	7	10.08
Opolskie	14	25.06	20	24.05	9	11.08
Warmińsko-Mazurskie	5	29.06	3	21.06	2	18.06
Podkarpackie	2	18.06	6	14.06	10	18.06

In the region of Wielkopolska (Poznań), in each year of observation, the occurrence of temperatures conditioning the development of these forms has been reported. This situation has been recorded continuously since 1999 (Ruszkowska 2007a). In 2007, in Opole (the Opolskie region), up to 20 days with a daily mean temperature of ≥ 25°C were recorded, which took place after 25 May.

In 2006, in the Warmińsko-Mazurskie region there were 5 days with temperatures ≥ 25°C, while for the first time in this region the occurrence of the infectious *R. padi* was recorded. Until then, among all the regions selected for study, no anholocyclic forms were found outside of the Podlaskie region.

The detection of BYD viruses

Detection of viruses in plants collected in fields

Results of the detection of BYD viruses in winter barley plants infested with aphids in the years 2006–2008, are presented in the table 3. In ELISA tests, the OD value in positive samples ranged from 0.1 to 0.9. In the case of a low virus concentration, where the results obtained in ELISA tests were doubtful, the molecular technique of RT-PCR was applied as a reference diagnostic method. Single RT-PCR products of the expected sizes: about 530 bp and 650 bp, respectively, from BYDV-PAV and BYDV-MAV infected plants were obtained (Figs. 2, 3).

Table 3. Detection of barley yellow dwarf viruses in winter barley plants collected in different regions of Poland in the years 2006–2008

Region and the date of sampling	Detection of viruses by ELISA (number of plants found infected/ number of plants tested)		
	BYDV-MAV	BYDV-PAV	CYDV-RPV
2006			
Opolskie, 26.10	0/21	8/21	–
Lubuskie, 12.11	0/12	12/12	–
Podkarpackie, 22.11	1/16	2/16	–
2007			
Opolskie, 18.10	0/25	21/25	–
Warmińsko-Mazurskie, 13.11	0/25	15/25	–
Podkarpackie, 22.11	4/25	7/25	–
Wielkopolskie, 3.12	0/25	5/25	–
Małopolskie, 6.12	2/25	14/25	
2008			
Opolskie, 4.04	11/25	5/25	8/25
Wielkopolskie, 20.10	0/16	11/16	0/16
Lubuskie, 23.11	2/16	4/16	0/16
Łódzkie, 7.11	11/32	14/32	7/32
Lubuskie, 13.11	1/32	32/32	1/32

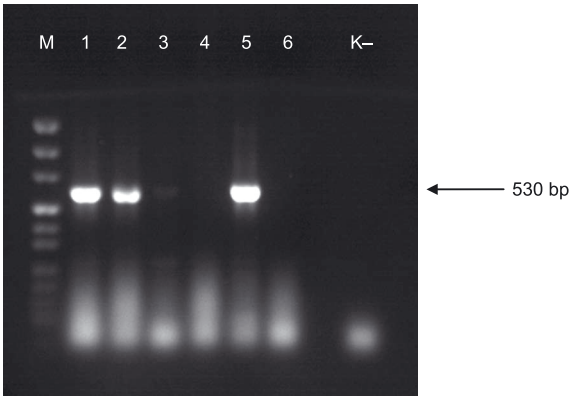

Fig. 2. The detection of BYDV-PAV in barley plants. Electrophoresis of the RT-PCR products using Lu1 and Lu4 primer pairs. M – O'Gene Ruler™ 100bp DNA Ladder, lanes:1, 2, 3, 4, 5, 6 – plant samples, K- – negative control

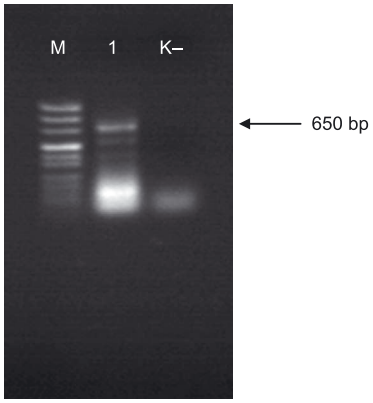

Fig. 3. The detection of BYDV-MAV in barley plants. Electrophoresis of the RT-PCR products using MAV3 and MAV4 primer pairs. M – O'Gene Ruler™ 100bp DNA Ladder; 1 – plant sample; K- – negative control

In all the years of our research, the dominating virus in barley fields was BYDV-PAV with the exception of the Opolskie region in spring 2008, when unexpectedly, the predominance of BYDV-MAV was revealed. In 2008, the diagnostics included the third potential causal agent of BYD, CYDV-RPV. The frequency of its occurrence, particularly in the Opolskie region was relatively high. On the basis of such a limited number of plants tested, it was not possible to point out a clear differentiation of the regions regarding the risk of BYD viruses incidence. The disease was encountered everywhere. However, from the obtained data, the most serious risk of BYD occurrence can be assumed to exist in the Opolskie region, where climatic conditions are especially favorable for aphid vectors.

The detection of BYD viruses in aphids

BYD viruses were easily detected in aphids using indirect procedures including test plant diagnostics. In each portion of aphids collected in winter barley fields under observation, the vectors of BYD viruses were detected. However, for the procedure applied, it was not possible to point out the proportion of BYD vectors in the general pool of aphids tested.

The direct procedure, RT-PCR, was successfully applied to detect BYDV-MAV and BYDV-PAV in their aphid vectors.

Twenty-five *R. padi* samples originating from 5 regions: Warmińsko-Mazurskie, Podkarpackie, Wielkopolskie, Dolnośląskie and Małopolskie, were analyzed.

In electrophoretical patterns the RT-PCR products of expected sizes, corresponding to the presence of the viruses and specific for aphids, were observed. Actin primers tested in RT-PCR with RNA from aphid samples, in all cases gave a single 320 bp product. Two distinguishable bands were visualised from viruliferous vectors and only the actin bands were found in the aviruliferous aphid samples. Electrophoresis of RT-PCR using primers sets: Lu1/Lu4 with Act1/Act2 and MAV3/MAV4 with Act1/Act2 were presented (Figs. 3, 4.). The presence of BYDV-MAV were confirmed in: 5 out of 5 tested field samples collected in the Warmińsko-Mazurskie, Podkarpackie, Dolnośląskie and Małopolskie regions, and 4 out of 5 analyzed samples from the Wielkopolskie region. BYDV-PAV was found in all the studied samples from the Dolnośląskie and Małopolskie regions, and 4 out of 5 assayed probes from the Podkarpackie and Wielkopolskie regions. There were no observed aphids originating from the Warmińsko-Mazurskie region with BYD viruses in their body.

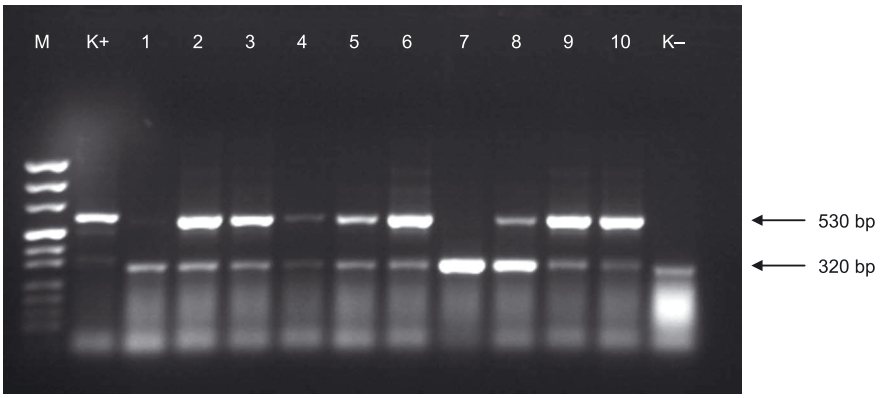

Fig. 4. The detection of BYDV-PAV in aphids. Electrophoresis of the RT-PCR products using Lu1/Lu4 and Act1/Act2 primer sets. M – O'Gene Ruler™ 100 bp DNA Ladder; K+ – positive control (infected aphids); 1, 2 3, 4, 5 – aphid samples from Wielkopolska; 6, 7, 8, 9, 10 – aphid samples from Podkarpackie; K- – negative control, virus free aphids

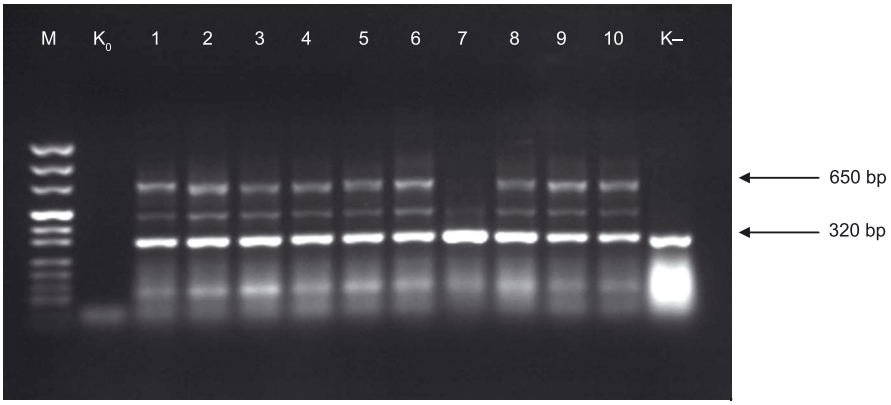

Fig. 5. The detection of BYDV-MAV in aphids. Electrophoresis of the RT-PCR products using MAV3/MAV4 and Act1/Act2 primer sets. M – O'Gene Ruler™ 100 bp DNA Ladder; K_0 – water control; 1, 2 3, 4, 5 – aphid samples from Małopolska; 6, 7, 8, 9, 10 – aphid samples from Wielkopolska; K- – negative control, virus free aphids

DISCUSSION

The course of temperature during the period of the research allowed for the designation of areas with expected occurrence of aphids on cereal crops - potential vectors of the Barley yellow dwarf viruses. The temperature occurrence which induces a change in aphid life cycle is very important, because the impact of photoperiod after 21 June already prompts the *gynoparae* development and greatly reduces the development of anholocyclic forms (Ruszkowska 2002).

The development of anholocyclic forms of aphids on winter crops in autumn is possible only under specific temperatures and could be a kind of a bio-indicator of global warming. On the basis of the temperature data, it can be concluded that anholocyclic forms which appear in different years and different regions of Poland, certainly come in large part from the native population. The development of anholocyclic forms is caused by two factors: the high temperature and quality (age) of the host plant (Ruszkowska 2002). On the secondary host, the anholocyclic forms of *R. padi* aphids develop in conditions of respectively high temperatures during spring and summer. In autumn the aphids feed on young plants, and this quality of host plants blocks the return to holocyclic development.

Direction of the territorial expansion of aphids-virus vectors on cereals is closely related to the temperatures in different regions.

The data concerning the detection of BYD viruses in autumn sown barley as well as in aphids infesting the fields are consistent with the data on the evolution of aphid population structure with the systematic increase of the anholocyclic forms (Jeżewska *et al.* 2010). However, the estimated number of aphids is not the only factor to be considered for the disease prediction. The proportion of aphid vectors of the BYD viruses in the general pool of aphids infesting fields, as well as the climatic conditions, mainly temperature, date of sowing, cultivars, and locations are also important factors for BYD risk predicting (McElphany *et al.* 1995; Bencharki *et al.* 2000). The detection of BYD viruses directly in aphids provides valuable information about the potential of infection pressure, provided it would be effective on a proper scale. The results of the research proved that this tool might be taken into account in special needs.

CONCLUSIONS

1. The development of anholocyclic forms of bird cherry-oat aphid, the main vector of barley yellow dwarf viruses, was obtained in all the studied regions except for the Podlaskie region.
2. The principal factor enabling the expansion of anholocyclic forms of *R. padi* was the increasing temperature.
3. Only the presence of virus vectors is not enough to prove plant infection. So far, the Polish populations of anholocyclic *R. padi* are only partial vectors, viruses were not found in the bodies of all aphids.
4. Common occurrence of BYD viruses in winter barley in autumn, confirms the risk of the disease which should be controlled.

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POLISH SUMMARY

ZAGROŻENIE ZBÓŻ WIRUSAMI ŻÓŁTEJ KARŁOWATOŚCI JĘCZMIENIA PRZENOSZONYMI PRZEZ ANHOLOCYKLICZNE FORMY *RHOPALOSIPHUM PADI* L. W POLSCE W LATACH 2006−2008

Wirusy żółtej karłowatości jęczmienia przenoszone są w sposób trwały głównie przez anholocykliczne formy mszycy czeremchowo-zbożowej (*Rhopalosiphum padi* L.). W Polsce gatunek ten dominuje na uprawach zbóż, a jego biologia związana jest ściśle z przebiegiem temperatur.

W latach 2006–2008 w celu określenia zasięgu występowania anholocyklicznych *R. padi*, a także oceny poziomu zagrożenia wirusami żółtej karłowatości jęczmienia, monitorowano 12 regionów Polski o zróżnicowanych warunkach temperaturowych. Obserwacje prowadzono pod kątem zasiedlania roślin przez anholocykliczne formy mszyc *R. padi*. Stwierdzono występowanie tych form na uprawach jęczmienia ozimego we wszystkich obserwowanych regionach, z wyjątkiem regionu podlaskiego.

W celu wykrywania trzech głównych sprawców żółtej karłowatości jęczmienia: *Barley yellow dwarf virus-MAV* (BYDV-MAV), *Barley yellow dwarf virus-PAV* (BYDV-PAV) i *Cereal yellow dwarf virus-RPV* (CYDV-RPV), próby roślin były poddane diagnostyce wirusologicznej. W 2007 roku przeprowadzono także badania na obecność wirusów żółtej karłowatości jęczmienia w mszycach żerujących na jęczmieniu ozimym. Wykazano, że najczęściej występującym wirusem był BYDV-PAV. Najwięcej mszyc – wektorów wirusów żółtej karłowatości jęczmienia, stwierdzono we wszystkich lokalizacjach regionu opolskiego, najcieplejszego regionu w Polsce.