https://doi.org/10.17221/52/2019-CJGPB

Reaction of winter wheat cultivars to eyespot assessed visually and by real-time PCR

Jana Palicová[1], Pavel Matušinsky[2,3], Alena Hanzalová[1], Ivana Svačinová[2], Veronika Dumalasová[1], Jana Chrpová[1]*

[1]Crop Research Institute, Prague, Czech Republic
[2]Agrotest Fyto, Ltd., Kroměříž, Czech Republic
[3]Department of Botany, Faculty of Science, Palacký University Olomouc, Olomouc, Czech Republic
**Corresponding author: palicova@vurv.cz*

Citation: Palicová J., Matušinský P., Hanzalová A., Svačinová I., Dumalasová V., Chrpová J. (2020): Reaction of winter wheat cultivars to eyespot assessed visually and by real-time PCR. Czech J. Genet. Plant Breed., 56: 9–14.

Abstract: The reaction of twelve winter wheat cultivars frequently grown in the Czech Republic and twenty-five new breeding lines to inoculation with *Oculimacula yallundae* and *Oculimacula acuformis* was evaluated in small plot trials from 2017–2018. The assessment was carried out visually by symptoms and by a quantitative real-time polymerase chain reaction (qPCR). The aims of the study were to compare the results of both methods, to evaluate the effect of the resistance gene *Pch1* to eyespot, and to select new breeding lines resistant to eyespot. The relationship between the eyespot symptoms and the pathogen DNA content in plant tissues followed a moderate linear regression. Low levels of eyespot were observed in the cultivars/lines possessing the resistance gene *Pch1* (Annie, Hermann, Rebell, SG-S1215-14, SG-S1825-14, SG-S791-13) and also in the line SG-SU630-15. The qPCR method was able to detect low levels of the pathogens in the plant tissue and to distinguish two eyespot pathogens. *O. acuformis* was detected in very low concentrations in the inoculated plants compared with *O. yallundae*. The eyespot infection rate was significantly higher in 2017 than in the next agricultural season due to extremely dry and warm spring weather in 2018.

Keywords: molecular markers; *Oculimacula acuformis*; *Oculimacula yallundae*; *Pch1* gene

Stem-base diseases of cereals are very important and can cause yield losses up to 40%. The complex of these diseases includes pathogens from different genera of fungi: *Oculimacula*, *Rhizoctonia*, *Fusarium*, *Microdochium* and *Gaeumannomyces*. Eyespot is thought to be the most serious disease from this group. It is caused by two species: *Oculimacula yallundae* (Wallwork & Spooner) Crous & W. Gams and *Oculimacula acuformis* (Nirenberg) Y. Marín & Crous. Both fungi seem to follow similar life cycles (Lucas *et al.* 2000). On the other hand, they differ in morphology, pathogenicity, occurrence and sensitivity to fungicides (Wei *et al.* 2011; Matušinsky *et al.* 2017). Eyespot pathogens have a wide host range among cereals and grasses. *O. yallundae* (OY) was prevalent in winter wheat samples attacked by eyespot in the Czech Republic (Palicová & Matušinsky 2019) whereas *O. acuformis* (OA) was predominant in winter rye eyespot samples in Lithuania (Ramanauskienė *et al.* 2014). *Oculimacula* spp. are supposedly able to survive on plant debris for more than 3 years and its occurrence varies due to the weather conditions (Matusinsky *et al.* 2008, 2009).

Conidia produced on infected straw are the principal form of the inoculum in the field (Leroux *et al.* 2012). Ascospores play an important role as a source

Supported by Ministry of Agriculture of the Czech Republic, Projects No. MZE-RO0418, MZE-RO1118 and QK1910041, QK1910197.

https://doi.org/10.17221/52/2019-CJGPB

of genetic variability within the pathogen populations, though they probably do not constitute a major source of the inoculum. Eyespot characteristically induces elliptical eye-shaped spots with diffuse margins on the leaf sheath at the base of the stem. They are usually highly visible at the stage of stem elongation. A visual assessment of the symptoms on the infected stems cannot discriminate between OY and OA and their presence can be masked by the less damaging pathogens of the stem base disease, especially early in the growing season (Turner *et al*. 1999, 2001). A real-time polymerase chain reaction assay, suitable for largescale testing, was developed and used for the quantitative detection and discrimination of OY and OA (Walsh *et al*. 2005).

There are four sources of eyespot resistance identified in commercial wheat varieties: *Pch1* (Worland *et al.* 1988), *Pch2* (de la Peña *et al.* 1996), *Pch3* (Murray *et al.* 1994), and a quantitative trait locus (QTL) *Q.Pch.jic-5A* (Burt *et al*. 2011). The most effective and also the most widely used resistance gene in commercial cultivars is *Pch1*, a single major gene mapped to the distal end of the long arm of chromosome 7D (Wei *et al.* 2011). It was transferred to wheat from *Aegilops ventricosa* (Maia 1967). Additional quantitative resistances on chromosomes 1A, 2B and 5D are known from cv. Cappelle-Desprez (Law *et al.* 1975). Resistance to eyespot was also identified in other wheat relatives including *Triticum tauschii*, *T. monococcum*, *etc*. (Cadle 1997; Figliuolo *et al.* 1998).

The aim of this study was to compare the visual assessment of eyespot and the assessment based on the quantitative real-time PCR (qPCR) and to carry out the quantitative detection and discrimination of OY and OA. The set of winter wheat cultivars and new breeding lines inoculated with *Oculimacula* spp. was assessed by both methods. The effect of the resistance gene *Pch1* to eyespot was also evaluated.

MATERIAL AND METHODS

Cultivar resistance. The reaction of twelve winter wheat cultivars and twenty-five new breeding lines to inoculation with OY and OA was studied in a small plot trial in Prague in 2016/2017 and 2017/2018. The set of the tested cultivars included cultivars registered in the Czech Republic, two of them (Annie, Rebell) possessing the eyespot resistance gene *Pch1* according to the molecular marker *Xorw1* analysis (Dumalasová *et al.* 2015). The new breeding lines selected to these trials were believed to be perspective based on the results of ring tests, where they demonstrated combined resistance. Only three lines were tested in both years (see Figure 2). Two of the lines were registered as varieties in the Czech Republic: Dancing Queen (SG-S1684-13) in 2018 and Illusion (SG-S1146-14) in 2019. The resistant controls were the cultivars possessing gene *Pch1* (Annie, Hermann) and the susceptible control was the cultivar Turandot.

The inoculum for the small plot trial was prepared from two isolates of OY and one isolate of OA. All the isolates were obtained from South Moravia (Kroměříž). The mycelium of the pathogens was grown on sterilised barley grains. The inoculum was applied on the experimental plots in November and in April (40 g/m^2). The reaction of all the cultivars was rated at the milk growth stage (BBCH 73-77). A 0 to 5 rating scale was used (0 – no symptoms, 1 – one small spot, 2 – more spots covering a maximally of half of the stem perimeter, 3 – spots covering more than a half of the stem perimeter, 4 – spot covering whole the stem perimeter, 5 – broken stem). In the inoculated plots, 15 randomly selected plants with 4 stems were assessed. The data were analysed by the UNISTAT 6.5 package (UNISTAT Ltd., London, UK) and Statistica 13.3 (Statsoft, Tulsa, USA) – by the Analysis of Variance (ANOVA), Multiple Comparison by Tukey and Regression Analysis.

Real-time PCR quantification of *Oculimacula* spp. The same set of winter wheat cultivars/lines from the experimental year 2018 inoculated with OY and OA was assessed visually as well as by qPCR. Twenty stem bases of each cultivar/line were collected randomly, afterwards dried and homogenised to a fine powder. The total genomic DNA was extracted using a DNeasy mericon Food Kit (Qiagen, USA). The extracted genomic DNA was run on electrophoresis in a 0.8% agarose gel to verify the quality. The concentration of the DNA was measured with Qubit (Termofisher Scientific, Waltham, USA) and the DNA was diluted to a concentration 10 ng/µl. The DNA samples were stored in a freezer under –30°C. The reverse primer Oculimacula-R universal to both OY and OA was used for the qPCR and the specificity between the two species was conferred by the forward primers Ac F-D (OA) and Yall F-H (OY) (Walsh *et al*. 2005). Phenylalanine ammonia-lyase (PAL) with the "WpalF" and "WpalR" primers of the wheat was used as a reference gene for the reaction normalisation (Walsh *et al*. 2005). The volume per qPCR reactions was 20 µl, comprising 10 µl of SYBR

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Table 1. The ANOVA results for the visual assessment of the wheat cultivars infected by eyespot (2017, 2018)

Effect	Sum of squares	Degrees of freedom	Mean squares	*F*	*P*
Intercept	4274.556	1	4274.556	2888.071	0.000 000
Cultivar	336.285	11	30.571	20.655	0.000 000
Year	551.306	1	551.306	372.486	0.000 000
Cultivar × year	71.069	11	6.461	4.365	0.000 002
Error	2095.783	1416	1.480		

Green qPCR Master Mix (Bio-Rad, USA), 7.5 µl of nuclease free water and 0.2 µl of (10 pmol/µl) primers and 2.0 µl of (10 ng) the analysed DNA. The qPCR reactions were conducted on a CFX96TM Real-Time PCR Detection System (Bio-Rad, USA). Plastic Low-Profile 0.2 ml 8-Tube Strips and Optical Flat 8-Caps (Bio-Rad, USA) were used for analysis. Three biological and three technical replicates were inspected. The initial denaturation temperature was 95°C, maintained for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. The primers specificity and presence of the primer dimers were verified by a melting analysis. The relative quantity was calculated using the $2^{-\Delta\Delta Ct}$ method based on threshold cycles of the reference and target genes with the CFX Manager 3.0 software (Bio-Rad, USA).

RESULTS AND DISCUSSION

Cultivar resistance. Statistically significant differences in the reaction of the tested winter wheat cultivars/lines to *Oculimacula* spp. inoculation were proven in the years 2017–2018 (Figures 1, 2). The cultivars/lines possessing the gene of resistance to eyespot *Pch1* mostly had less disease symptoms than the others without *Pch1*. These symptoms on the resistant cultivars/lines with *Pch1* were nonspecific necrotic spots on the stem bases different from typical elliptical eye-shaped spots. The cultivars Rebell and Annie (both with the gen *Pch1*) showed the least disease symptoms in the tested set of cultivars in both years. The cultivar Bohemia was the least infected variety without the *Pch1* gene, followed by Matchball, Tobak, Vanessa, Arkeos, Genius, Turandot (susceptible control), Julie, Patras and Dagmar in 2017 (Figure 1A). The average infestation of the cultivar Dagmar almost reached stage 3 in 2017, it means the stem-based spots covered more than half of the stem perimeter. In 2017, the infection rate varied between 1.1 (recorded for the cultivar Annie) and 2.9 (the cultivar Dagmar). The differences in the two agricultural seasons were statistically significant (Table 1).

In 2018, the infection pressure achieved was lower than in 2017 (Figure 1B). It ranged from 0.3 (the cultivar Rebell) to 1.9 (the cultivar Julie). The great differences in the eyespot symptoms intensity between the two years were caused by the extremely dry and warm weather of spring 2018. The spring conidia production at an optimum temperature of 10°C was crucial on the plots inoculated with eyespot at the

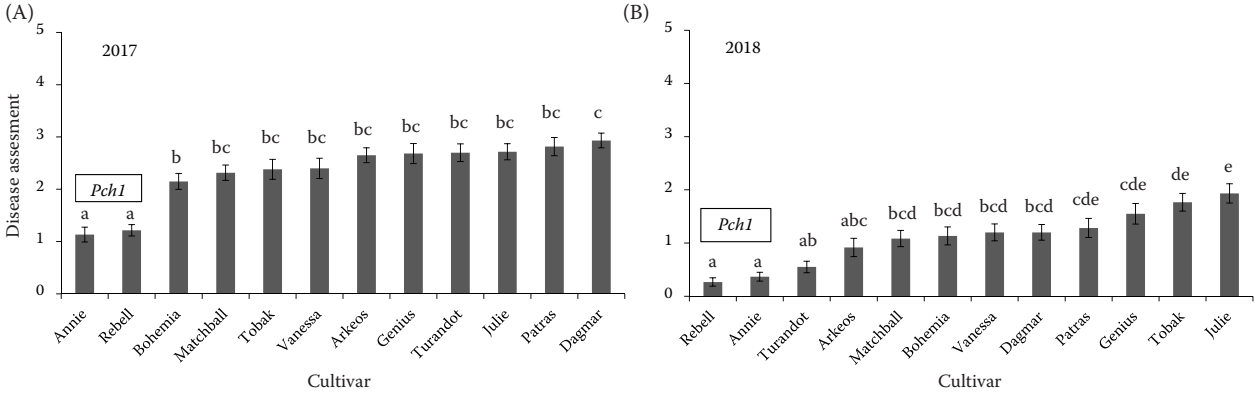

Figure 1. The visual assessment of the eyespot symptoms in the winter wheat cultivars in 2017 (A) and 2018 (B)
The analysis of variance (ANOVA) with a multiple comparison Tukey test ($P < 0.05$); the homogeneous groups are marked with letters (the same letter – is not significantly different from each other)

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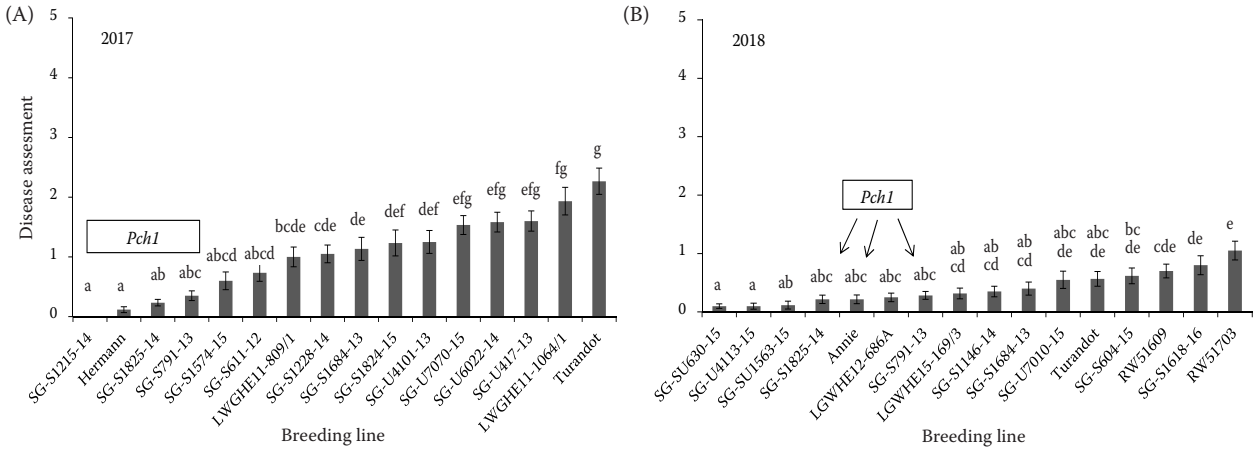

Figure 2. The visual assessment of the eyespot symptoms in the winter wheat breeding lines in 2017 (A) and 2018 (B) The ANOVA with a multiple comparison Tukey test ($P < 0.05$); the homogeneous groups are marked with letters

end of November. However, there was a very short period with temperatures near 10°C in 2018. In March, the average temperatures in Prague had been below zero for several days, and the temperatures rose above 15°C in April. The conidia production was, therefore, much lower than in the previous year. Moreover, the inoculated plots suffered from drought. The cultivar Turandot (susceptible control) showed a surprisingly very low level of eyespot symptoms (0.6) in 2018. The greatest eyespot symptoms were found in the cultivar Julie (1.9) but the reaction is thought to be rather moderately resistant to moderately susceptible.

On average, the set of the tested new breeding lines showed an approximately one-degree lower eyespot infection than the set of the tested cultivars in both years. The lines differed significantly and showed a resistant reaction. Almost no symptoms were found in the three lines with the *Pch1* gene, similar to the resistant cultivar Hermann in 2017 (see Figure 2A). No material has reached the infection value of the susceptible control cultivar Turandot. The evaluated eyespot symptoms were lower in 2018 than in 2017 (Figure 2B) similar to the set of the tested cultivars. The cultivar Turandot reached a very low infection value (0.6) and four lines were attacked more by eyespot than Turandot, but only up to the value 1 (resistant reaction).

The statistically significant difference between the eyespot symptoms of the genotypes carrying the *Pch1* gene of the resistance and the genotypes without the *Pch1* was obvious in this study. In Germany, it has been consistently described that the cultivars possessing *Pch1* gene have been characterised by high

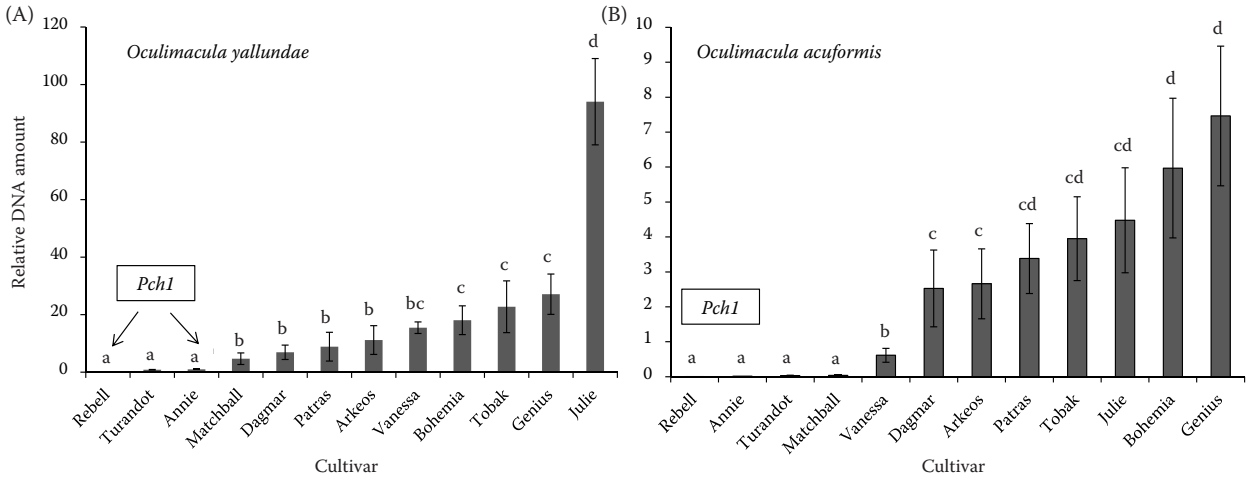

Figure 3. The qPCR assessment of *Oculimacula yallundae* (A) and *O. acufomis* (B) in the winter wheat cultivars (2018) The analysis of variance with a multiple comparison Tukey test ($P < 0.05$); the homogeneous groups are marked with letters

https://doi.org/10.17221/52/2019-CJGPB

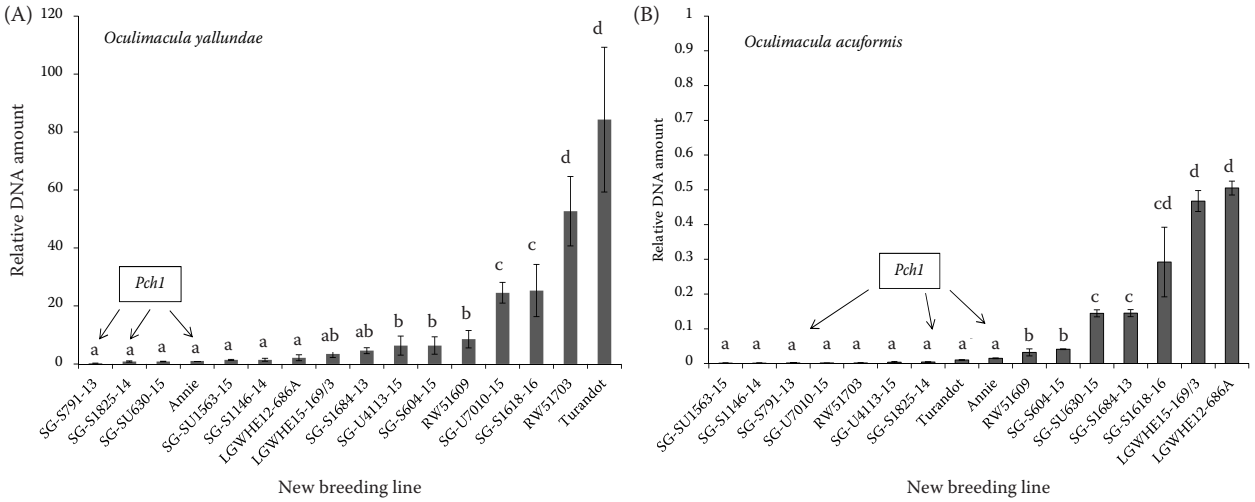

Figure 4. The qPCR assessment of *Oculimacula yallundae* (A) and *O. acufomis* (B) in the winter wheat breeding lines (2018) The analysis of variance with a multiple comparison Tukey test ($P < 0.05$); the homogeneous groups are marked with letters

resistance to eyespot in field conditions (Meyer *et al*. 2011). No isolate of *Oculimacula* spp. obtained from France was found virulent on the *Pch1* carrying genotypes (Saur & Cavelier 1995).

Real-time PCR quantification of *Oculimacula* spp. *Oculimacula* spp. quantification based on the qPCR analysis and its comparison to the visual assessment of the disease severity on the plants in the small plot field experiment was the main task of this study. The most resistant cultivar Annie (carrying the gene *Pch1*) was set as the control and the relative amount of *O. yallundae* DNA in the other varieties measured by the qPCR was related to this control sample as a fold difference (FD). In the case of *O. yallundae*, which was more frequently found in the samples and in a higher amount, the lowest DNA level was determined in the cultivars Rebell (*Pch1*, FD 0.09), Turandot (FD 0.85) and Annie (*Pch1*, FD 1). The highest level of DNA was detected in the cultivars Julie (FD 94.03), Genius (FD 27.11), Tobak (FD 22.76) and Bohemia (FD 18.07) (Figure 3A). These results correspond with the visual assessment in 2018 (Figure 1A). *O. acuformis* was detected in the samples with much lower intensity than *O. yallundae*. A higher amount of this species was detected in the same cultivars as above and Genius (FD 7.46), Bohemia (FD 5.97) Julie (FD 4.48) and Tobak (3.95), the lowest DNA level was found in the cultivars Rebel (FD 0.006), Annie (FD 0.01) and Turandot (0.03) (Figure 3B). In the experiment with the new breeding lines, the highest level of OY was detected in Turandot (FD 84.29), RW51703 (FD 52.69) and SG-S1618-16 (FD 25.31), the lowest level in the two lines carrying the *Pch1* gene SG-S791-13, SG-S1825-14 (Figure 4A). OA was detected in a very low level (FD < 1) in this experiment (Figure 4B).

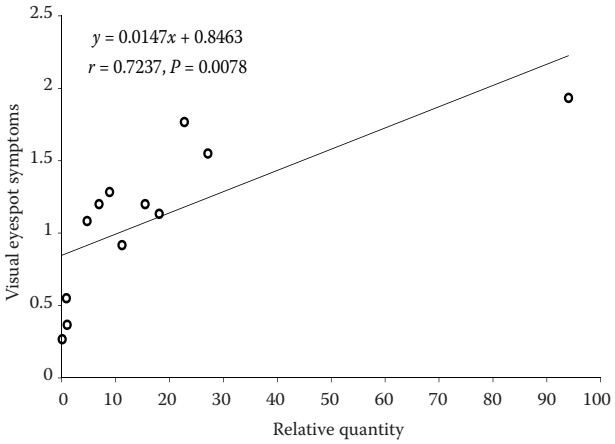

Figure 5. The relationship of the visual assessment and the relative quantity obtained during the qPCR (2018)

The linear regression analysis indicates that the relationship of the visual assessment and relative quantity obtained during the qPCR showed a correlation coefficient 0.7237 ($P = 0.0078$), indicating a strong relationship between the symptoms and the pathogen DNA content in the plant tissues (Figure 5).

According to our results, the qPCR method can be applied for eyespot diagnostic assays including a wheat cultivar resistance assessment. This sensitive method can better distinguish the small differences among the tested materials than the visual assessment even in the years unfavourable to the disease

https://doi.org/10.17221/52/2019-CJGPB

development. Moreover, the level of the pathogens can be checked by qPCR easily. This molecular method can be useful for a long breeding process which completes the registration of a new variety.

Acknowledgements. We would like to thank L. Urbánková, I. Rutrlová and Š. Bártová for the technical support.

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Received for publication May 31, 2019
Accepted after corrections September 10, 2019
Published online October 25, 2019