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RESEARCH ARTICLE

Alternaria alternata (Fr.) Keissl with mutation G143A in the Cyt b gene is the source of a difficult-to-control allergen

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Abstract The saprotrophic fungus *Alternaria alternata* is widespread in the agro-environment and produces more than ten allergenic proteins, mostly protein Alt a 1. The frequency of the *Alt a 1* gene was analyzed in a group of *A. alternata* isolates from winter wheat kernels obtained in Poland, and the effectiveness of various fungicides targeting the pathogen was evaluated. The *Alt a 1* gene was identified in four of the seven tested isolates. *A. alternata* colonized 35.67% kernels on average, but its frequency increased in stored grain where the presence of epiphytes was noted on 23.09 to 51.38% kernels, and endophytes—in 26.21 to 42.01% of kernels. The efficacy of field-applied fungicides did not exceed 50%, despite the fact that *A. alternata* is highly sensitive to propiconazole, fenpropimorph, and tebuconazole under in vitro conditions. The analyzed isolates were characterized by limited sensitivity to azoxystrobin (EC_{50} ranged from 0.505 to 1.350 μg cm^{-3}) due to a mutation at codon 143 of the *CYT b* gene, responsible for resistance to quinone outside inhibitor fungicides, which was noted in all isolates. The spread of *A. alternata* can be effectively controlled with suitable fungicides and by monitoring the prevalence of pathogenic isolates in the environment.

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Introduction

Asthma is a common chronic disease that affects around 334 million of people around the world (Global Asthma Report 2014). Each year, undiagnosed and untreated asthma leads to premature death in up to 250,000 cases (Bousquet et al. 2010). The prevalence of asthma is highest among children aged 6 to 14 years (up to 22%), and the disease affects every tenth adult (Barnes et al. 2001). Asthma should be monitored from an early age. The prevalence of asthma in children differs significantly in Eastern European countries, and it is higher in Poland than in Belarus and the Ukraine (Brozek et al. 2016). According to the cited authors, the prevalence of the disease is determined by the quality of health services and the effectiveness of diagnosis and treatment.

According to Samoliński et al. (2007), transient or persistent symptoms of allergic diseases, including nasal congestion, allergic asthma, and atopic dermatitis, affect 30–40% of the Polish population. Nasal symptoms are present in 6 to 85% of patients with asthma, and they are a risk factor for developing the disease (Leynaert et al. 1999, Brozek et al. 2017). Amirmajdi et al. (2011) observed a significant correlation between allergic rhinitis and the presence of *Alternaria* in the nasal cavity (Amirmajdi et al. 2011). The spores of the *Alternaria alternata* (Fr.) Keissl fungus are one of the key causes of allergy (Dadas-Stasiak et al. 2010). IgE-dependent hypersensitivity was reported in more than 95% of patients allergic to *A. alternata* (Chruszcz et al. 2012; Twaroch et al. 2012). The allergens present in *A. alternata* spores or in dog or cat hair trigger identical allergic reactions. However, *A. alternata* spores are very difficult to eliminate because they

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are freely disseminated by wind, and they directly reach the human respiratory tract (Knutsen et al. 2012). *A. alternata* spores may be particularly dangerous for agricultural employees and persons and come into direct contact with grain contaminated by this fungus (Skóra et al. 2015).

A. alternata is a ubiquitous saprotroph which colonizes cereal spikes under supportive weather conditions to cause sooty head mold (Toklu et al. 2008). This pathogen has a broad spectrum of host plants which, according to the USDA Systematic Botany and Mycology Fungus-Host Distribution Database (http://nt.ars-grin.gov), included 674 plant species in 2017. In addition to sooty head mold, *A. alternata* also causes other diseases, such as leaf spot, leaf rot, and leaf blight.

The fungus produces more than ten allergenic proteins (Gabriel et al. 2016; Kustrzeba-Wójcicka et al. 2014). The most widely researched protein Alt a 1 (AAM90320.1, www.ncbi.nlm.nih.gov/, NCBI, Protein Database) is associated with asthma (Gabriel et al. 2016; Hong et al. 2005). Alt a 1 is a glycoprotein with molecular weight of 30 kDa. A crystallographic analysis conducted by Chruszcz et al. (2012) demonstrated that Alt a 1 is a unique β-barrel protein comprising 11 β-strands that form a dimer. Monomers are linked by a single disulfide bond. This unique structure is characteristic of a probably new family of proteins detected exclusively in the kingdom Fungi (Chruszcz et al. 2012). The biological functions of protein Alt a 1 in fungal cells have not yet been fully elucidated. The protein probably participates in the transport of small molecules and plays important roles during the maturation of fungal spores (Wagner et al. 2014).

Sooty head mold occurs in cereal spikes mainly in periods of heavy rainfall which delays harvest. Black soot-like growth appears on infected spikes (Toklu et al. 2008). The affected heads exhibit extensive, dark olive-green and black discoloration. Head mold infects plants that are deficient in nutrients, lodged, or mechanical damaged. The prevalence of sooty mold increases in wet weather which is favorable for fungal growth (Hershman 2011). Triazole and strobilurin fungicides are not highly effective against *A. alternata* (Wang et al. 2002). Infected cereals can be a significant reservoir of allergens, in particular in extensive cropping systems. In general, triazole drugs target the ergosterol biosynthetic pathway by inhibiting lanosterol 14 alpha demethylase (CYP51), a key enzyme encoded by the ERG11 gene (Zou et al. 2014). Preparations such as quinone outside inhibitors (QoI) suppress mitochondrial respiration in fungal cells by binding to the ubiquinone coenzyme in cytochrome b and c1 (enzyme complex III). They block the transport of electrons between those cytochromes, which reduces ATP production and depletes energy reserves in cells (Gisi et al. 2002; Bartlett et al. 2002).

The aim of this study was to determine the potential of *A. alternata* isolates to synthesize Alt a 1 protein, to analyze the prevalence of *A. alternata* on winter wheat grain, to evaluate in vitro the effectiveness of fungicides applied during the growing season of winter wheat, and to identify *A. alternata* forms resistant to QoI fungicide azoxystrobin.

Materials and methods

Origin of winter wheat grain

Winter wheat grain was obtained from a field-plot experiment conducted in north-eastern Poland (53.713 N, 20.361 E) in 2010–2012. Winter wheat cv. Bogatka (List of cultivars… 2010) was grown on plots with an area of 25 m^2. The experiment had a randomized block design with four replications. Commercial fungicides were applied two or three times during the growing season in stages BBCH 31 (beginning of stem elongation), BBCH 55 (heading), and BBCH 71 (watery ripe stage) (Meier 2003), with a backpack sprayer (Marolex, Titan 12, Poland), according to the schedule presented in Table 1. Unprotected plots were the control. All agricultural treatments were applied in accordance with GAP standards for winter wheat. The entire experimental period was characterized by mild winters, and ambient temperatures did not fall below − 9 °C in the coldest years of 2010 and 2012. Moderate temperatures (19 °C) and moderate precipitation (80.4 mm) were noted before harvest in July 2010. In the remaining years of the study, total July precipitation was somewhat higher (202.8 mm in 2011 and 121.0 mm in 2012), and average temperatures were somewhat lower (17.6 and 17.7 °C, respectively). High precipitation levels before harvest contributed to the development of *A. alternata*.

Isolation of fungi from grain

The counts of *A. alternata* were determined at harvest and in wheat grain stored for 6 months in paper bags at a temperature of 11 °C (Table 2). Epiphyte colonies were cultured by placing 10-g grain samples in 250-cm^3 Erlenmeyer flasks filled with 90 cm^3 of sterile water. To isolate endophytic fungi penetrating kernels, identical grain samples were disinfected for 10 min with 1% NaOCl (Abchem, Poland), ground in an impact mill (Predom, Poland), and placed in 250-cm^3 flasks filled with 90 cm^3 of sterile water. The flasks were shaken for 30 min at 180 rpm on the shaker table (Elpin plus 358 S, Poland). Three-fold (10^{-3}) diluted suspensions of fungal CFU were plated on Petri dishes (Ø = 90 mm) in the amount of 1 cm^3 and immersed in selective Martin's agar medium (White et al. 1990; Martin 1950) cooled to a temperature of 42 °C. To determine the prevalence of *A. alternata* in winter wheat grain (in Table 2), non-surface disinfected kernels (for epiphyte counts) and kernels disinfected for 10 min in 1% NaOCl (for endophyte counts) were plated on potato dextrose agar with pH 5.5 (PDA, Merck, Poland) with the addition of

Table 1 Pesticide treatments applied to winter wheat

Treatment	BBCH 31	BBCH 55	BBCH 71
Economical 1 (ECON-1)	Alert 375 SC[1] (flusilazole 125 g/dm^3, carbendazim 250 g/dm^3)	No treatment	Tarcza Łan 250 EW[2] (tebuconazole 250 g/dm^3)
Intensive 1 (INT-1)	Corbel 750 EC[3] (fenpropimorph 750 g/dm^3)	Opera Max 147,5 SE[4] (pyraclostrobin 85 g/dm^3, epoxiconazole 62.5 g/dm^3)	Tarcza Łan 250 EW[2] (tebuconazole 250 g/dm^3)
Intensive 2 (INT-2)	Corbel 750 EC[3] (fenpropimorph 750 g/dm^3)	Opera Max 147,5 SE[4] (pyraclostrobin 85 g/dm^3, epoxiconazole 62.5 g/dm^3)	Topsin M 500 SC[10] (thiophanate-methyl 500 g/dm^3)
With growth regulator 1 (REG-1)	Moddus 250 EC[5] (trinexapac-ethyl 250 g/dm^3)	Amistar 250 SC[6] (azoxystrobin 250 g/dm^3)	Topsin M 500 SC[10] (thiophanate-methyl 500 g/dm^3)
With growth regulator 2 (REG-2)	Moddus 250 EC[5] (trinexapac-ethyl 250 g/dm^3)	Amistar 250 SC[6] (azoxystrobin 250 g/dm^3)	Tarcza Łan 250EW[2] (tebuconazole 250 g/dm^3)
Biotechnological (BIOT)	Asahi SL[7] (*o*-nitrophenol 2%, *p*-nitrophenol 3%, 5-nitroguaiacol 2%)	Biochicol 020 PC[8](chitosan)	Biochicol 020 PC[8] (chitosan)
Economical 2 (ECON-2)	No treatment	Bumper 250 EC[9] (propiconazole 25.1%)	Topsin M 500 SC[10] (thiophanate-methyl 500 g/dm^3)

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Table 2 Colonization of winter wheat grain by *A. alternata*

Treatment*	CFU × 10 ^{2} in 1 g of grain†					Percentage of kernels colonized by the pathogen‡				
	Epiphytes		Endophytes		Mean	Epiphytes		Endophytes		Mean
	At harvest	After 6 months	At harvest	After 6 months		At harvest	After 6 months	At harvest	After 6 months	
Control	6.66	11.33	7.00	10.33	8.83	$27.77^{a–e}$	$47.22^{a–e}$	$29.16^{a–e}$	$43.06^{a–e}$	36.81
ECON-1	7.00	14.66	8.67	10.33	10.16	$29.17^{a–e}$	61.11^{a}	$36.11^{a–e}$	$43.05^{a–e}$	42.36
INT-1	3.33	12.33	4.54	8.00	7.08	13.88^{e}	$51.38^{a–e}$	$19.44^{c,d,e}$	$33.33^{a–e}$	29.51
INT-2	4.66	12.00	6.33	4.33	6.83	$19.44^{c,d,e}$	$50.00^{a–e}$	$26.38^{a–e}$	$18.06^{c,d,e}$	28.47
REG-1	7.66	8.66	6.33	8.67	7.83	$31.94^{a–e}$	$36.11^{a–e}$	$26.38^{a–e}$	$36.11^{a–e}$	32.63
REG-2	5.00	13.33	5.33	11.33	8.75	$20.83^{b–e}$	$55.55^{a,b,c}$	$22.22^{a–e}$	$47.22^{a–e}$	36.45
BIOT	5.66	14.00	5.66	13.33	9.67	$23.61^{a–e}$	$58.33^{a,b}$	$23.61^{a–e}$	$55.55^{a,b,c}$	40.27
ECON-2	4.33	12.33	6.33	14.33	9.34	$18.06^{c,d,e}$	$51.38^{a–e}$	$26.39^{a–e}$	$59.72^{a,b}$	38.88
Mean	5.54^{z}	6.29^{z}	10.08^{y}	12.33^{y}	8.56	23.09^{x}	51.38^{y}	26.21^{x}	42.01^{y}	35.67
Year		2010	2011	2012			2010	2011	2012	
		5.56^{B}	8.53^{B}	11.59^{A}			23.17^{B}	35.54^{A}	11.59^{C}	

A–B for years; x, y, z for date × epiphyte/endophyte; a–e for treatment × date × epiphyte/endophyte

*Refer to Table 1 for the legend

† Obtained by washing off CFU from kernel surface onto Martin's agar

‡ Obtained by plating 100 kernels on PDA. Values in columns that did not differ significantly in the SNK test ($p < 0.01$) are marked with identical letters

the antibiotics kanamycin and streptomycin (Sigma, Poland). The number of *A. alternata* colonies was counted after 7 days of incubation at 24 °C.

Identification of fungi

Preliminary fungal isolation was carried out under a light microscope (Nikon Eclipse E 200, Japan) at ×400 magnification. The morphological characteristics of *A. alternata* were determined. Multicellular spores formed chains on straight or branching dark olive conidiophores ending in scars. Thick-walled spores were cylindrical in shape, with transverse and longitudinal septa and characteristic short hyaline hyphae. The appearance and size of most spores (20–63 μm in length, 9–18 μm in width) corresponded to those described by Ellis (1971). Molecular identification was performed with the use of a pair of primers that amplify the non-coding region between rRNA genes. DNA was isolated from fungal filaments of seven endophytic isolates of *A. alternata* (1P, 3P, 5P, 6, 7, 8, and 8P) obtained from stored grain. The fungal filaments were cultured on PDA (Merck, Poland) for 48 h, and they were transferred to a liquid medium (0.1% beef extract, *w/v*, 0.5% soy peptone, *w/v*, 0.5% sodium chloride, *w/v*, 1% glucose, *w/v*, 0.7% yeast extract, *w/v*, distilled water) in 50-cm^3 flasks. Fungal DNA was isolated with the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. The quantity and quality of DNA was checked by measuring absorbance at a wavelength of 230 and 260 nm (NanoMaestro Gen, Poland). The polymerase chain reaction (PCR) was performed according to the procedure described by White et al. (1990) with the use of the following pair of primers: ITS5 forward 5′-GTAT CGGACGGAGATCCAGC-3 and ITS4 reverse 5′-TTGC TCAGTGCATTGTCGG-3′. Reaction products were separated by electrophoresis on 1% agarose gel (Prona, Poland) with ethidium bromide (EtBr, Sigma, Poland). Amplification products were sequenced at the Institute of Biophysics and Biochemistry of the Polish Academy of Sciences in Warsaw (https://www.ibb.waw.pl). Fungal isolates were identified by comparing the similarity of sequences with the BLAST tool in the NCBI database (NCBI 2017).

Detection and identification of the Alt a 1 gene

The sequence encoding the *Alt a 1* gene was detected and identified by PCR. The amplified sample of 50 μL contained 34.7 μL of sterile ddH_2O, 5 μL of 10× PCR buffer (Sigma, Poland), 3 μL of $MgCl_2$ (25 mM, Sigma, Poland), 4 μL of dNTP (10 mM of all nucleotides, 2.5 mM of each nucleotide, Sigma, Poland), 0.5 μL of the Alt-forward primer (5′-ACGA GGGTGAYGTAGGCGTC-3′), 0.5 μL of the Alt-reverse primer (5′-ATGCAGTTCACCACCATCGC-3′), 0.3 μL of Taq polymerase (Sigma, Poland) (5 U/μL), and 5 μL of DNA. Amplification was carried out in the Mastercycler Ep Gradient thermocycler (Eppendorf, Poland) with the following thermal profile: preliminary denaturation at 94 °C (4 min), followed by 35 cycles of: denaturation at 94 °C (40 s), hybridization at 57 °C (30 s), elongation at 72 °C (10 min), and final extension at 72 °C (10 min). Amplicons with the size of 510 bp were visualized in a transilluminator (UVP, Poland).

In vitro evaluation of the sensitivity of *A. alternata* mycelia to fungicides

The effectiveness of Amistar 250 SC, Topsin M 500 SC, Tarcza Łan 250 EW, Bumper 490 EC, and Corbel 750 EC fungicides was evaluated in three replications. PDA disks with 7-day-old colonies of *A. alternata* (1P, 3P, 5P, 6, 7, 8, and 8P isolates) were plated on Petri dishes (Ø = 90 mm) on PDA with the addition of the examined fungicides. Fungicides were applied at the following concentrations of the active ingredient, 0.001, 0.01, and 0.1 μg/cm^3. Petri dishes containing PDA without the fungicides were the control. The colonies were incubated in darkness for 4 days, they were scanned (Epson Perfection scanner, USA), and their surface area was calculated in the ImageJ 1.49 program (Rasband 1997). The results were log transformed, graphically interpolated, and used to calculate the EC_{50} values of the tested fungicides (expressed in micrograms per milliliter).

Examination of the sensitivity of *A. alternata* spores to azoxystrobin

A. alternata colonies cultured for 7 days at 24 °C in darkness were exposed to UV light (TL-D 360 W/08 BLB, 360–380 λ nm, Philips, Poland) to stimulate sporulation. The spore suspension was brought to a concentration of 3×10^5 conidia in cubic centimeter with a hemocytometer. The Amistar 250 SC fungicide was serially diluted in water to produce concentrations of 0.1, 1.0, and 100.0 μg/cm^3. Aliquots of 0.1 cm^3 of every fungicide solution were placed on microscope slides and combined with 10 μl of the suspension of *A. alternata* spores. The number of germinating spores was counted under a microscope (Nikon E200, Japan) after 48 h of incubation in a moist chamber at a temperature of 24 °C. Spores where the length of filaments was at least equal to spore length were regarded as germinated. The EC_{50} values determined for each isolate represented effective azoxystrobin concentrations which inhibited spore germination by 50% on average in six replications. The results were log transformed, and EC_{50} values were determined by graphing fungicide concentrations with the 50% value on the *x*-axis (expressed in micrograms per milliliter).

Analysis of point mutations at codon 143 of the CYT b gene of *A. alternata* isolates

Spontaneous point mutations in the cytochrome b gene (*CYT b*) responsible for strobilurin resistance were detected in the mismatch amplification mutation assay with the use of the FailSafe PCR system (Epicenter, USA). Isolates sensitive to azoxystrobin were analyzed with the use of StroSNP2fwd (5′-CTTATG GTCAAATGTCTTTATGATG-3′) and StrobSNP1rws (5′-GGTGACTCAACGTGATAGC-3′) primers, and the obtained fragment had a length of 639 bp. A 302-bp fragment of resistant isolate was amplified with the use of StrobSNPrcF7 (5′-CAAT AAGTTAGTTATAACTGTTGCGG-3′) and StrobSNPrcR1 (5′-CTATGCATTATAACCCTAGCGT-3′) primers (Sigma, Poland). Isolates resistant and sensitive to azoxystrobin were identified in two separate reactions according to the protocol described by Siah et al. (2010).

Statistical analysis

The number of fungal CFU eluted from grain in 1 cm^3 (N) was calculated with the use of the following formula:

$$N = \frac{n}{10^{-r} \times v} \quad (1)$$

where:

n	Number of plated colonies
10^{-r}	Dilution coefficient
v	Volume of plated suspension

The analysis of variance was performed in the Statistica 12 program (StatSoft Inc 2014), and the significance of differences between means was estimated in the SNK test ($p < 0.01$). The sequences of the experimental isolates and the reference isolates in the NCBI database were compared in the MEGA 6 program to assess their evolutionary relatedness (Tamura et al. 2013). The phylogenetic tree was generated by the neighbor-joining (NJ) method. Data were verified with the use of the Jukes-Cantor model. The reliability of phylogenetic trees was evaluated by bootstrapping with 2000 replications.

Results

Fungicide efficacy against sooty head mold

A. alternata colonized 35.67% of wheat kernels on average (Table 2). The prevalence of *A. alternata* increased significantly during grain storage (from 23.09 to 51.38% for epiphytes and from 26.21 to 42.01% for endophytes). Intensive fungicide treatment (INT-1) with tebuconazole was most effective, and it reduced the number of epiphyte *A. alternata* CFU on grain by 50% (immediately after harvest) in comparison with control. The applied fungicides had no significant effect on grain colonization by *A. alternata* or the number of CFU per gram of grain. The prevalence of *A. alternata* ranged from 13.88% (INT-1) to 61.11% (ECON-1), and the number of CFU × 10^2 per grain of grain ranged from 3.33 (INT-2) to 14.66 (ECON-1) (Table 2).

A significant positive correlation was noted between the percentage of colonized kernels and the number of CFU ($r = 0.835$, $p < 0.01$). It should be noted that the prevalence of *A. alternata* increased significantly after 6 months of grain storage—more than 2.2-fold for epiphytes (from 23.09 to 51.38%) and more than 1.6-fold for endophytes (from 26.21 to 42.01%). July precipitation levels were significantly correlated with the number of *A. alternata* CFU on grain ($r = 0.867$, $p < 0.01$). High precipitation levels in July of 2011 (202.8 mm) and July of 2012 (121.0 mm) enhanced the growth of *A. alternata* on grain (8.53 and 11.59 × 10^2 CFU, respectively), and a dry spell in July 2010 (80.4 mm) inhibited grain colonization by *A. alternata* epiphytes (5.56 × 10^2 CFU).

In vitro evaluation of the sensitivity of *A. alternata* isolates to fungicides

In most cases, fungicides containing thiophanate-methyl, tebuconazole, propiconazole, and fenpropimorph completely inhibited the development of all tested *A. alternata* isolates in vitro already at the lowest concentration ($EC_{50} < 0.001$ μg/cm^3) (Table 3). The only exceptions were isolates 3P and 5P which were less sensitive to thiophanate-methyl (EC_{50} 0.052 and 0.160 μg/cm^3, respectively). Only two isolates, 3P and 6, responded to azoxystrobin by 50% inhibition of colony growth at concentrations of 0.019 and 0.106 μg/cm^3. The EC_{50} values of the remaining isolates, estimated based on colony growth inhibition, exceeded 0.100 μg/cm^3. The EC_{50} values in the spore germination assay ranged from 0.505 μg/cm^3 for isolate 3P to 1.350 μg/cm^3 for isolate 5P (Fig. 1).

Identification and phylogenetic analysis of *A. alternata*

All analyzed isolates produced spores characteristic of *A. alternata,* and an analysis of sequences in conserved regions ITS 1 and ITS 2 confirmed that the identified isolates belonged to the species *A. alternata.* The degree of similarity of all analyzed nucleotide sequences ranged from 98 to 100% (Fig. 2). An analysis of a cladogram developed based on the nucleotide sequences of the experimental and reference isolates of *A. alternata* revealed that the experimental isolates (1P, 3P, 5P, 6, 7, 8, and 8P) and selected reference isolates (KP267527, KP267523, KP267528, KT362732, KU319070, KU319071) were grouped within the same clade (Fig. 2). The other clade contained two *A. alternata* isolates (isolate 8P and

Table 3 The efficacy of commercial fungicides (expressed as EC_{50} of isolate) in controlling mycelial growth in studied *A. alternata* isolates (μg/cm^3)

Isolate Fungicide	1P	3P	5P	6	7	8	8P
Thiophanate-methyl	< 0.001	0.052	0.160	< 0.001	< 0.001	< 0.001	< 0.001
Azoxystrobin	> 0.100	0.019	> 0.100	0.106	> 0.100	> 0.100	> 0.100
Propiconazole	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Fenpropimorph	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Tebuconazole	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

EC_{50} = effective concentration needed to reduce fungal growth by 50%

reference isolate KM215626). The isolate of *Rhizoctonia cerealis* (AF222793) belonged to an outgroup.

Presence of the *Alt a 1* gene marker and a comparison of experimental and reference isolates

The PCR product with an estimated length of 510 bp, obtained with the application of the *Alt a 1* gene marker, was detected in four *A. alternata* isolates: 1P, 3P, 5P, and 6 (Fig. 3). These isolates harbored the gene encoding allergenic protein Alt a 1. In those isolates, the sequences of conserved ITS regions were identical to the corresponding sequences in reference isolate ATCC 6663 harboring the *Alt a 1* gene.

Presence of mutations at codon 143 of the CYT b gene in *A. alternata* isolates

The presence of allele G143 or A143 encoding sensitivity or resistance to azoxystrobin, respectively, was analyzed in a fragment of the cytochrome b gene (Fig. 4). In seven resistant isolates containing allele A143, the PCR product was a single amplicon with an estimated length of 302 bp. Amplicons with the size of 639 bp indicative of azoxystrobin sensitivity were not detected in any *A. alternata* isolates.

Discussion

In this study, the *Alt a 1* gene was identified in most of the *A. alternata* isolates colonizing winter wheat grain. In a study by Skóra et al. (2015), the *Alt a 1* gene was present in all tested isolates belonging to the genus *Alternaria*, and those isolates synthesized Alt a 1 protein regardless of the applied culture medium. Hong et al. (2005) detected the *Alt a 1* gene in 52 species of *Alternaria* and related genera. A very high number of *A. alternata* spores containing the gene encoding allergenic protein Alt a 1 poses a significant health risk for persons working in an environment where grain is handled and processed. Spores are transported by wind across hundreds of kilometers, and they can also be found in areas that are located remotely from fields and storage facilities (Ogórek et al. 2011). Grisoli et al. (2009) demonstrated that the concentrations of airborne *A. alternata* spores were highest in the direct vicinity of storage facilities and decreased with distance from those sites. On dry, warm, and windy days in summer, in particular during harvest, the number of *A. alternata* spores in grasslands and cereal fields can range from 500 to 1000 spores per m^3, whereas the number of spores in indoor premises generally ranges from 3 to 1000 spores per m^3 (Skóra et al. 2015; Brito et al. 2012; Vijay et al. 1999).

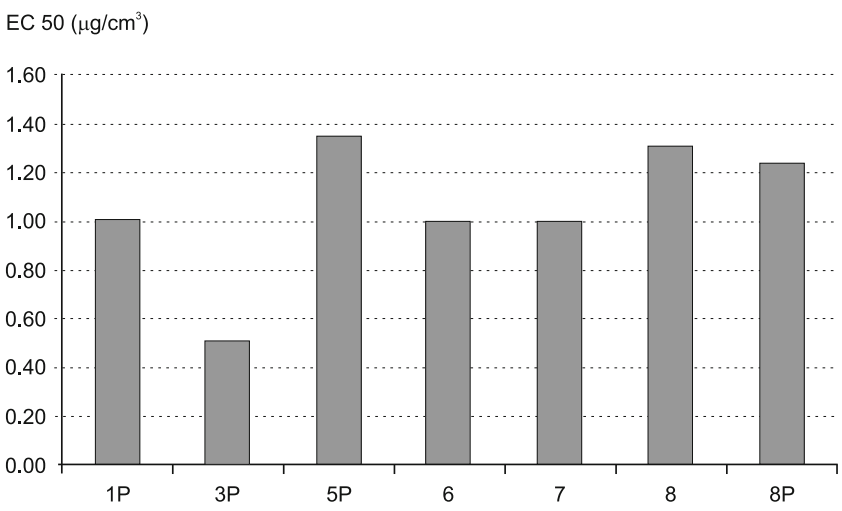

Fig. 1 The efficacy of commercial azoxystrobin fungicide (expressed as EC_{50}) in controlling spore germination of resistant *A. alternata* isolates

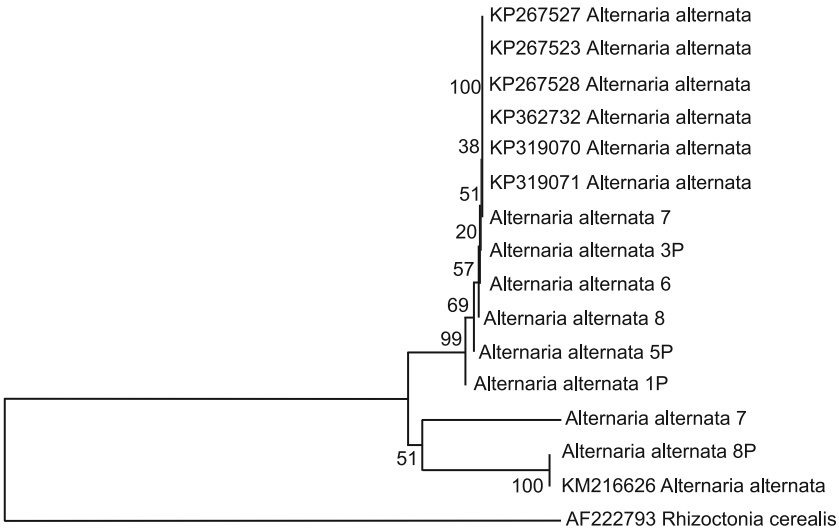

Fig. 2 A phylogenetic tree of *A. alternata* isolates created with the neighbor-joining (NJ) method. The NJ method reflects the degree of phylogenetic affinity between the identified fungal species. Pathogens were identified with ITS4 and ITS5 markers. The reliability of the phylogenetic tree was evaluated by bootstrapping with 2000 replications and bootstrap support > 70%

In the present study, *A. alternata* colonized 35.67% kernels on average, and its prevalence was positively correlated with precipitation levels in July. Similar results were reported by Patel and Minipara (2015). In the work of Nuray (2005), the prevalence of *A. alternata* on wheat grain was somewhat lower in the range of 0–23%. The prevalence of *A. alternata* was determined at 60–100% by Hudec and Muchova (2008) and at 35.4–48% by Casa et al. (2012).

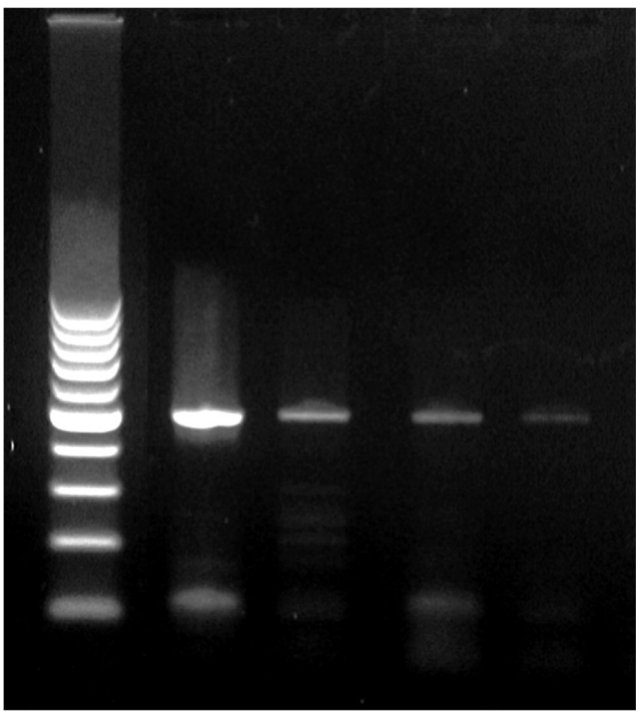

Fig. 3 An image of a gel post-electrophoresis. EtBr was added to the gel before electrophoresis to visualize products with the expected size of 510 bp. Line 1: marker 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp; Lines 2–6: *A. alternata* 1P, 3P, 5P, and 6

In our study, the abundance of *A. alternata* increased 1.6- to 2.2-fold (depending on the isolation method) after 6 months of grain storage. In the work of Casa et al. (2012), the abundance of *A. alternata* was reduced by up to 57.1% after 6 months of grain storage. However, according to many authors, *A. alternata* is the predominant pathogen capable of colonizing up to 85% of stored grain (Barkat et al. 2016; Kulik et al. 2014; Patriarca et al. 2007).

The inhibition of *A. alternata*, the main cause of sooty head mold in cereals, could reduce the number of allergenic spores in air. However, in our study, the effectiveness of intensive fungicide treatments in inhibiting fungal colonization did not exceed 50% relative to unprotected plants. Casa et al. (2012) demonstrated that the effectiveness of triticonazole and iprodione preparations and difenoconazole fungicides was only somewhat higher at 55.1 and 44.9%, respectively.

In Central Europe, QoI fungicides have been used to protect cereal crops for more than 25 years. However, *Blumeria graminis* f. sp. *tritici* (wheat powdery mildew) isolates resistant to QoI fungicides were found in Germany already in 1988 (Bartlett et al. 2002). To date, resistance to QoI fungicides has been identified in *Alternaria* spp. populations colonizing various crops, including tomatoes (Chapin et al. 2006), potatoes (Pasche et al. 2004), pears and apples (Lu et al. 2003), tangerines (Vega and Dewdney 2014), and pistachios (Ishii 2010; Ma et al. 2003). Vega and Dewdney (2012) analyzed 148 isolates of *A. alternata* isolates from citrus trees and found that 68% of those isolates were resistant to QoI fungicides. In our study, *A. alternata* isolates from wheat grain were resistant to azoxystrobin in both phenotypic and molecular analyses. The EC_{50} values of azoxystrobin and the tested isolates ranged from 0.019 to > 0.100 μg/cm^3 in the fungal colony test and from 0.505 to 1.350 μg/cm^3 in the spore germination test. In a study of isolates obtained from citrus trees, Vega and Dewdney (2012) observed that discrimination of isolates

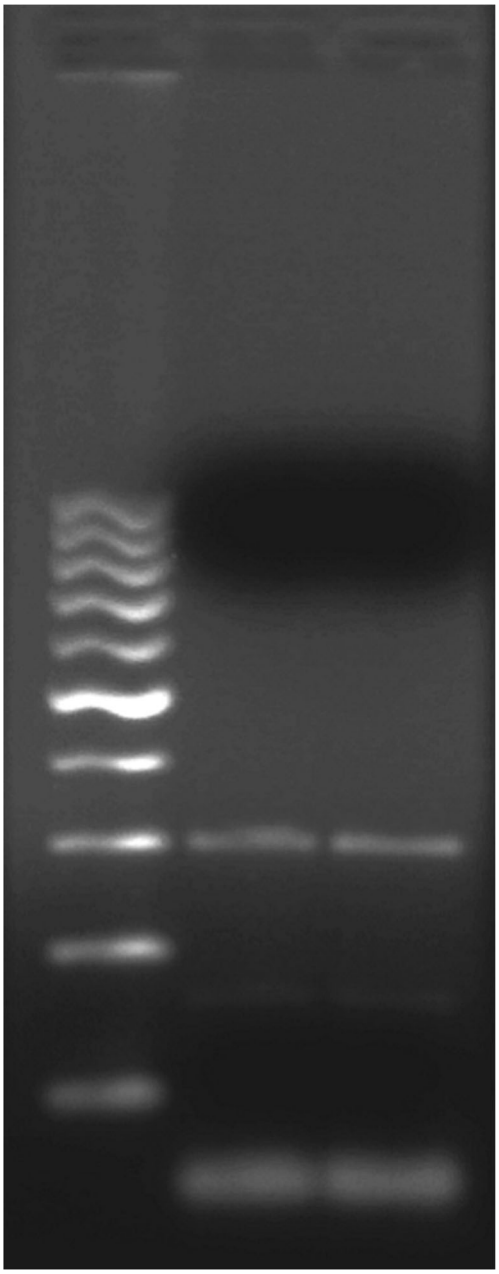

Fig. 4 An image of a gel post-electrophoresis. EtBr was added to the gel before electrophoresis to visualize products with the expected size of 302 bp. Line 1: marker 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp, Lines 2, 3: *A. alternata* 1P and 3P

sensitive to azoxystrobin takes place at a concentration of 5 μg/cm^3. In the cited study, the EC_{50} values of sensitive isolates (resazurin-based microtiter test versus spore germination assay) ranged from 0.017 to 0.192 μg/cm^3. When Mondal et al. (2005) conducted a baseline sensitivity study for *A. alternata* in citrus fruit, they found EC_{50} values greater than 100 μg/ cm^3 for azoxystrobin based on mycelial growth inhibition tests, which demonstrated that *A. alternata* mycelium is insensitive to azoxystrobin. The EC_{50} values for *A. alternata* isolates colonizing cereal grain have not been determined to date. It is worth noting that QoI fungicides exert considerable pressure on *A. alternata* colonizing cereal crops in Central Europe because these fungicides have been widely applied in this region since 1998. Nearly 15 years ago, Bartlett et al. (2002) demonstrated that azoxystrobin effectively inhibited spore germination and the growth of *A. alternata* mycelia on wheat leaves. Based on the published data, *A. alternata* isolates from wheat are unlikely to have developed resistance to QoI fungicides by a point mutation—substitution of alanine for glycine at codon 143 in the *Cyt b* gene (G143A). The mechanism of resistance to QoI fungicides in field crops usually relies on a point mutation at codon 143 (Gisi et al. 2002; FRAC Code List 2016; Sierotzki et al. 2000). In our study, the mutation at codon 143 was noted in *A. alternata* isolates from wheat grain. The above mutation was also reported in *A. alternata* isolates from potatoes (Hausladen et al. 2015), but not in *Alternaria solani* isolates from tomatoes where F129L substitutions where found (Odilbekov et al. 2016).

Conclusion

High precipitation before wheat harvest facilitates grain colonization by *A. alternata* whose spores may contain the gene encoding allergenic protein Alt a 1. Intensive fungicide treatments are only partially effective in limiting the growth of this pathogen, and morpholine and triazole fungicides deliver the best results. Excessive use of QoI fungicides discriminates *A. alternata* isolates with point mutation G143A which conditions resistance to those crop protection agents. The spread of sooty head mold can be effectively controlled with suitable fungicides and by monitoring the prevalence of pathogenic isolates in the environment. Regular monitoring of harvested and stored grain is critical for minimizing the health risks posed by fungal allergens.

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