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Spreading of *Alternaria* spp. in Mycoflora of Winter Wheat Seeds in North-East of Ukraine

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Abstract: The article deals with the abiotic and biotic factors which influence the dynamic system of wheat seed mycoflora. The study of seed-born fungi in the north-east of Ukraine showed the dominance of a genus of fungi, *Alternaria*. The diagnosis of species according to the peculiarities of colony growth on potato-carrot environment and the structure of conidial sporulation, 5 kinds of fungi were discovered (*A. tenuissima* (Nees et T. Nees: Fr.), *A. alternata* (Fr.) Keissl. *A. infectoria*, *A. arborescens* and *A. avenicola* E.G. Simmons, Kosiak & Kwasna). *A. arborescens* (37.4%) was the most common species among those identified. PCR analysis of experimental survey confirmed the dominance of this species in the north-east of Ukraine. The investigation of the spreading of the genus of *Alternaria* proved their placing in five regions from the north to the south with the largest amount of fungi in the northern territories. It can be concluded that the presence of *Alternaria* species varied depending on the place of sampling and the year of study.

Keywords: *Alternaria* species, Seed mycoflora, Winter wheat, The north-east of Ukraine

The mycoflora of seeds consists of different species of fungi, the presence of which is determined by various factors. The main world representatives of the complex of wheat seed fungi were *Alternaria*, *Aspergillus*, *Ceratobasidium*, *Cercospora*, *Cochliobolus*, *Curvularia*, *Drechslera*, *Fusarium*, *Gaeumannomyces*, *Microdochium*, *Penicillium*, *Pyricularia*, *Pythium*, *Rhizoctonia*, *Rhizopus*, *Sclerophthora*, *Trichoderma* and *Tricoconella* (Miller 1995). The fungi of the *Alternaria* genus are widespread among the seed mycoflora of cereals: in Argentina (Andersen et al 2015), Italy (Ramires et al 2018), Germany (Müller and Korn 2013), Slovakia (Mašková et al 2012), Russia (Gannibal 2018), Kazakhstan (Turzhanova et al 2020). During the last ten years, several studies of *Alternaria* spp. in wheat seeds have been done by Ukrainian scientists. During 2009-2012 only *A. alternata* (20.3%) was isolated in the endophytic mycoflora of wheat seeds in the central part of the country (Kovalishina et al 2012). The analysis of isolates of *Alternaria* spp. in different regions of Ukraine during 2012-2013 showed the dominance of *A. tenuissima* (70%) and a significant percentage of *A. infectoria* (25.6%) (Holosna 2015). In the region of Polissya during 2011-2013 only one species *(A. alternata)* was isolated (35.6%). It dominated in the mycoflora of wheat seeds (Tymoshchuk et al 2014). Due to its wide distribution, the genus of *Alternaria* plays a significant role as in wildlife so in the human life. Most *Alternaria* species are saprophytes, which along with other microorganisms, destroy plant debris. *Alternaria* fungi were the main endophytes of seeds and stems of *Triticum aestivum* L., *T. dicoccoides* and *Aegilops sharonensis* (Ofek-Lalzar et al 2016). They produce about 300 secondary metabolites that have dangerous (phytotoxins, mycotoxins). *Alternaria* fungi can cause allergic reactions in humans (Kttafah et al 2020). Some species produce substances with herbicidal (Dalinova et al 2020), insecticidal (Singh et al 2012), antimicrobial (Berestetskiy et al 2018) and antiviral (Bashyal et al 2014) properties. In China, *A. alternata* was isolated from rust pustules of *Puccinia striiformis* f. sp. tritici and proved to be a hyperparasite of this biotrophic wheat pathogen (Zheng et al 2017). *Alternaria* fungi are dangerous phytopathogens that cause diseases of economically important plants, including cereals, fruits, oilseeds, cucumbers, tomatoes, citrus, cauliflowers, peppers, tobacco, and strawberries etc. (Lee et al 2015, Meena et al 2016). Thus, *Alternaria* spp. has a significant distribution in the mycoflora of seeds in Ukraine and other countries. The variability of their species composition has been confirmed by various researchers. There is no monitoring of *Alternaria* fungi in Ukraine. Therefore, the aim of the study was to establish their distribution among other fungi of wheat seeds mycoflora and determine their species composition.

MATERIAL AND METHODS

Analysis of seed-born fungi: Thirty samples of winter

wheat seeds of Ukrainian and foreign selection were collected from five regions of north-eastern Ukraine during 2018–2019: Shostka (51.76921234521743, 33.48084672807519), Hlukhiv (51.67418850582228, 33.9098918820434), Sumy (50.88382108560319, 34.771726091851214; 50.93410032452191, 34.78592051269139), Okhtyrka (50.21895647608306, 35.02756103561299), and Kharkiv (49.994852637197205, 36.452404689801284) regions. The analysis of seed mycoflora was performed by biological methods on potato-glucose agar (PGA) (Kyryk and Pikovsky 2012). Two hundred seeds were taken from each sample. First, they were rinsed for an hour under running water and then sterilized in 1% potassium permanganate solution for 1-2 minutes and washed with sterile water. Subsequently, they were dried in two layers of filter paper and 20-25 seeds were placed into Petri dishes. Incubation was performed in a thermostat at a temperature of 22-24°C. The seeds were examined for the presence of seed borne mycoflora from the third to the eighth day of incubation. Fungi were identified using a microscope (morphology of mycelium, asexual and sexual sporulation).

Identification of *Alternaria* spp.: *Alternaria* fungi were sowed in a special environment (potato-carrot agar, PCA). Petri cups were incubated in special lighting conditions for a photoperiod of 12/12 hours under fluorescent lamps. Fungi were identified by sporulation habit and morphology of conidia and conidiophores (Simmons 2007, Gannibal 2011).

PCR diagnostics of *A. arborescens*: DNA isolation from samples of the genus of *Alternaria* was performed using the NeoPrep100 DNA Magnet_plant set (NEO_GEN, Ukraine). This set is based on Lysing soln. It is intended for celllysis, solubilization of cell debris, as well as for denaturation of cell nucleases. DNA sorbs on NeoSorb (R) (sorbent) in the presence of Lysing soln. Then it easily washed off the proteins and the salt of Buffer soln. DNA can be used for its intended purpose without further purification or processing. Lysis was performed in a 3 mol solution of guanidine thiocyanate (produced by Amresco, USA) at 65°C per 1 hour. PCR was performed using a set of reagents for DNA amplification "PCR MIX 2x HOT" (produced by NEO_GEN, Ukraine), which contains a ready-made mixture for DNA amplification (Taq DNA polymerase, which was inhibited for "hot start"; deoxynucleoside triphosphates, $MgCl_2$, paint for application to the gel as part of an optimized buffer system for standard PCR). The total volume of the reaction mixture was 20 µl (10 µl of 2x mixture for PCR; 0.1 µl of specific primers; 4.9 µl of deionized water for PCR, 5 µl DNA). Sequence of specific primers: direct – 5'GCTCACTCGATTGC ATGCACCTCA3'G, reverse – 5'TGTTGCTCATT TCGGATGCTG3'. The amplification program included the following stages: 95°C for 12 minutes – 1 cycle, 95°C for 15 seconds – 40 cycles, 65°C for 15 seconds – 40 cycles, 72°C for 15 seconds – 40 cycles, 72°C for 1 minute – 1 cycle. PCR products were separated by electrophoresis in 2.5% agarose gel at a voltage of 8 V/cm (30 minutes) with ethidium bromide.

RESULTS AND DISCUSSION

Identification of *Alternaria* species by cultural and morphological characteristics: Initially, the colonies of *Alternaria* fungi, which grew in wheat seeds during the analysis of seed mycoflora on potato-glucose agar environment were observed. The colonies differ in colors, structures and intensities of air mycelium formation. Different formations of sporulation of fungi from Petri dishes were noted with a microscope. A detailed study of conidia showed their different structures. Therefore, a special environment (Potato Carrot Agar, PCA) was used to identify the following species: *A. tenuissima, A. alternata, A. infectoria, A. arborescens,* and *A. avenicola*. The main features of the identification of small-spore species were the three-dimensional sporulation patterns and the structure of conidia (Fig. 1).

A. tenuissima on PCA formed colonies with a dark center. Similar colonies of this species were grown in isolation from soybeans, but they still had separate dark areas (Stevan et al 2011). Conidia were arranged in long chains (5-17). Spores are brown with a short secondary

Fig. 1. The three-dimensional sporulation pattern of *Alternaria* spp. on PCA (*A. tenuissima*, *A. alternata*, *A. arborescens*, and *A. avenicola)*

conidiophore, often had 3-4 longitudinal and 2 transverse septas. Lateral secondary conidiophores were formed on the parts of the conidia. *A. alternata* had almost black colonies on the PCA. The color, structure, and the growth of colonies of this species depend on many factors (Mishra and Khan 2014). Depending on the environment, their color can be varied from pale white to black (Dipak et al 2013). Conidia are more elongated in comparison with *A. tenuissima*. There were 4-6 conidia in one short chain. Spores formed small bushes and had an apical secondary conidiophore with 1 conidiogenic locus.*A. infectoria* formed light brown colonies with light aerial mycelium in the center on the PCA. A typical feature of this species complex was the late formation of conidia (7-10 days). Spores had light brown color, atypical shape. The presence of long secondary conidiophores in conidia was noted. The three-dimensional sporulation pattern differs from simply broken chains to the characteristic large bushes. *A. arborescens* had several types of colonies. In most cases the fungi of the PCA formed gray-green colonies with gray aerial mycelium in the center. Then small *alternata* and *A. tenuissima* (Ramires et al 2018). The cluster of *A. alternata* and *A. tennuissima* was dominant in wheat seeds with black point in Kazakhstan (Turzhanova et al 2020).

Determination of *A. arborescens* by PCR: Since the isolation spectrum of *Alternaria* spp. has changed in recent years (emergence of new species and their dominance in the north-eastern forest-steppe of Ukraine) additional PCR diagnostics were performed. Twenty isolates, which we previously identified as *A. arborescens* by the characteristics of conidial sporulation. Isolates of two species were included into the study (*A. avenicola* (Hlukhiv district) and *A. alternata* (Okhtyrka district)) to compare the results. The affiliation of all studied isolates to the species of *A. arborescens* (except for two) was confirmed (Fig. 2).

The belonging of the isolates of Kharkiv region (X1, X2, X5) to *A. arborescens* was confirmed. X4 and isolates aa1 and av1 (*A. alternata* and *A. avenicola*, respectively) were not representatives of this species. B2, CM7, P1 and KSh2 (the first three variants of Sumy and the fourth one of Shostka

chains (5-6 brown spores) attached to the long conidiophores, which did not branch much. In some cases there was the formation of dark green colonies, in which the chains of spores were more often branched. Conidia had characteristic of secondary apical conidiophores. Apical branching characteristic of *A. arborescens* was observed even on PGA in 24-hour darkness. Although previous studies have shown the increase in lateral and suppression of apical branching when this species was grown in complete darkness (Orina et al 2010). *A. avenicola* of KMA had brown colonies with gray aerial mycelium. Conidia are light brown and of various shapes and spore production is quite abundant. A characteristic feature was the presence of spores with lateral secondary conidiophores, which formed short chains or individual conidia. In most cases they were formed on opposite parts of the same conidia.

Thus, most of the identified species belong to small-spore *Alternaria* species, which have a conidia length of less than 60 µm. The species *A. alternata, A. arborescens* and *A. tenuissima* were phylogenetically very close and combined into one section of *Alternaria* (Gannibal 2015). The species of the *A. infectoria* complex are included in the *Infectoria* section (Lawrence et al 2016). *A. avenicola* belongs to the section of *Panax* (Woudenberg et al 2013). Therefore, fungi of the *Alternaria* section are dominated in the mycoflora of winter wheat seeds in the north-eastern forest-steppe of Ukraine during 2018–2019. Recent analyzes of the species composition of *Alternaria* fungi have also shown the dominance of this section. In Italy, 105 isolates out of 134 belonged to the *Alternaria* section, and most were *A.* regions) were identified as isolates of *A. arborescens*. Isolates of *A. avenicola* and *A. alternata* did not show affiliation to the studied species. The determination of the species of *A. arborescens* according to the structure of conidial sporulation and morphology of colonies on KMA was confirmed by PCR.

Spreading of *Alternaria* spp. in the internal mycoflora of winter wheat seeds: The mycoflora of winter wheat seeds was studied on potato-glucose agar (PGA). The genus of *Alternaria* was dominated among the other species in the complex of fungi inside the seeds for two years of research. Among five identified *Alternaria* species, only one was predominated (*A. arborescens* -Table 1). Other species have not been identified in other regions during the years of research. Only once *A. avenicola* had a significant percentage of isolation in Hlukhiv region in 2019, but it was widespread in all samplings. In contrast to the previous studies (Rozhkova et al 2016) with the dominance of *A. tenuissima* in the north-eastern Forest-Steppe, present

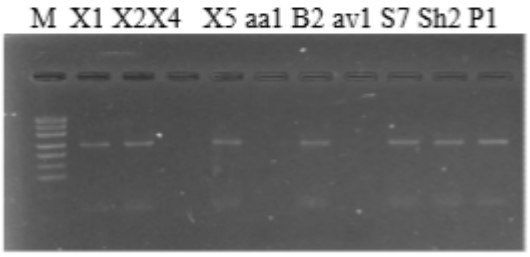

Fig. 2. PCR results on the electrophoregram

investigation proved the distribution of it in three regions. Its highest percentage of isolation was noted in Okhtyrka region. *A. infectoria* was distributed in four regions with a low percentage (maximum was 9%). *A. alternata* had the lowest distribution and amount of isolation.

The place of cultivation of winter wheat significantly affected the allocation of *Alternaria* species in different years by 5% less level of probability ($p<0.0497$). Similar results were obtained by Gannibal (2018). Infection of wheat and barley grain in different years on average in different districts of Krasnodar and Stavropol Territories varied significantly for the *Alternaria* section 7.0-71.5% and *Infectoriae* – 8.6-74.0%. In most cases, the contamination of grain simultaneously in different regions and in different years in the same region differed statistically significantly ($p<0.001$). The study of the mycocomplex of winter wheat seeds from different areas of Ukraine in 2016–2017 showed a decrease in the presence of *A. alternata* from North to South. This species was contained in all analyzed samples from Polissya, from the Forest-Steppe – in 56% of samples, from the Steppe – in 40% (Ostrovsky et al 2018). Isolation of different *Alternaria* species was found when growing wheat in different places. In 2018, three different species were isolated from wheat seeds in Shostka, Hlukhiv, and Sumy regions. Two species were identified in the Okhtyrka region, and four species – in the Kharkiv region. In 2019, the number of species in three places increased (Shostka, Hlukhiv, and Okhtyrka regions). The same species remained in Sumy, their quantity decreased to two in Kharkiv region. Thus, *A. arborescens* was dominated in all regions where seed samples were taken. Its highest percentage of isolation was 63.9% in Hlukhiv region (2018). Its prevalence averaged is 37.4% over two years of research. *A. avenicola* is significantly inferior to the previous species in its amount of 10.5%. *A. tenuissima* and *A. infectoria* had a low percentage

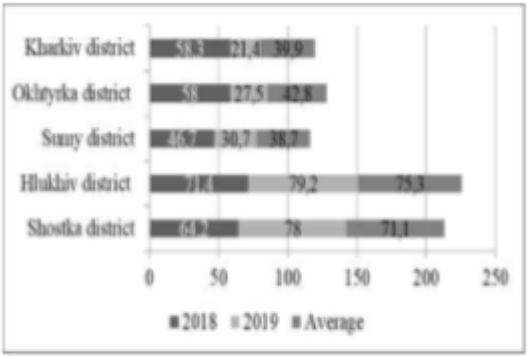

Fig. 3. Presence of *Alternaria* spp. in the internal mycoflora of winter wheat seeds ($p=0.865$ (2018); $p=0.025$ (2019)

of prevalence among the other species (3.4 and 2.1%, respectively), and *A. alternata* had very minor one. The calculation of the total amount of *Alternaria* sp. proved their dominant presence in the northern regions, such as Hlukhiv and Shostka (Fig. 3).

The total number of *Alternaria* spp. isolated from seeds significantly depended on the place of cultivation of wheat only in 2019. The highest percentage of fungal isolation was noted in 2019. The lowest indicators of the presence of *Alternaria* fungi were determined in the same year in the southernmost region of Kharkiv. On average, the percentage of isolation of the genus of *Alternaria* sp. makes about 53.6% during 2018-2019 in the north-eastern Forest-Steppe of Ukraine. This indicates their dominance among other fungi of the winter wheat seed mycoflora. Studies of other Ukrainian scientists have also proved the dominant position of the genus of *Alternaria* sp. in Sumy and Kharkiv regions (Mykhalska et al 2019).

Table 1. Species of the genus *Alternaria*, which are parts of the internal mycoflora of winter wheat seeds (north-east of Ukraine)

Place of seed sampling	Species of the genus *Alternaria*, % isolation among all fungal colonies				
	A. arborescens	*A. avenicola*	*A. tenuissima*	*A. alternata*	*A. infectoria*
	2018/2019	2018/2019	2018/2019	2018/2019	2018/2019
Shostka region	54.9/57.1	0.5/16.8	8.5/1.5	-*	-/2.6
Hlukhiv region	63.9/12.5	-/64.5	5/2	-	2.5/0.3
Sumyregion	35.3/29.5	5.1/0.9	-	-	6.3/0.3
Okhtyrka region	41.1/24.3	-/1.7	16.9/-	-/1.5	-
Kharkiv region	35/20.3	14/1.1	-	0.3/-	9/-
On the average	46/28.7 (37.4)	3.9/17 (10.5)	6.1/0.7 (3.4)	0.06/0.3 (0.2)	3.6/0.6 (2.1)
LSD (0.05)	4.2/2.5	3.2/1.9	2.3/ -	-	1.6/0.5

Note: *- Fungi didn't germinate from seeds

CONCLUSION

The average isolation of *Alternaria* fungi from winter wheat seeds was 53.6%. Therefore, they predominated in the mycoflora among others during 2018-2019 in the north-eastern Forest-Steppe of Ukraine. Five species of *Alternaria* fungi were identified in potato-carrot environment: *A. tenuissima*, *A. alternata*, *A. infectoria*, *A. arborescens*, and *A. avenicola*. PCR identification of *A. arborescens* confirmed the correctness of the preliminary species determination. *A. arborescens* (37.4%) was the most common species among those identified. The small-spore species from the *Alternaria* section had a dominant position in the mycoflora of winter wheat seeds. The region of cultivation significantly affected the amount of isolation and representativeness of *Alternaria* species. These fungi showed their dominant presence in the northern regions such as Hlukhiv and Shostka. The highest percentage of fungal isolation was in 2019 (79.2%).

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