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DYNAMICS OF MAIZE PATHOGENS FROM FUSARIUM, ASPERGILLUS AND PENICILLIUM GENERA IN SOIL UNDER WEATHER CONDITIONS OF REPUBLIC OF MOLDOVA

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The results of molecular expertise of phytosanitary state of upper layer of soil from experimental cornfields are presented. Several fungal pathogens from *Fusarium*, *Aspergillus* and *Penicillium* genera were identified using PCR assay. These pathogens were identified in soil samples during the three-year monitoring period and their respective quantities depended on weather conditions. Abiotic factors like air temperature and relative humidity have a significant impact on fungi's propagation. Weather conditions of the year affected significantly accumulation of *F. equiseti, F. graminearum, A. clavatus, P. expansum* and *P. chrysogenum* in soil. A positive correlation between pathogens' quantities and values of relative air humidity was found.

Keywords*: soil, maize, fungi, pathogens, Fusarium*, *Aspergillus*, *Penicillium, PCR.*

DINAMICA PATOGENILOR AI PORUMBULUI DIN GENURILE FUSARIUM, ASPERGILLUS ŞI PENICILLIUM ÎN SOL SUB ACŢIUNEA FACTORILOR CLIMATICI AI REPUBLUCII MOLDOVA

În lucrare sunt prezentate rezultatele analizei stării fitosanitare a stratului superior de sol de pe câmpurile experimentale de porumb. Unii patogenii din genurile *Fusarium*, *Aspergillus* şi *Penicillium* au fost identificaţi prin metoda PCR. Speciile respective au fost identificate în sol pe parcursul perioadei de monitorizare timp de trei ani şi cantităţile lor respective au fost afectate de condiţiile climatice. Factorii abiotici precum temperatura aerului şi umiditatea relativă au un impact semnificativ asupra propagării ciupercilor. Condiţiile anului au afectat semnificativ propagarea în sol a *F. equiseti, F. graminearum, A. clavatus, P. expansum* şi *P. chrysogenum*. A fost identificată corelaţia pozitivă dintre cantitatea patogenilor în sol şi valorile umidităţii relative a aerului.

Cuvinte-cheie*: sol, porumb, ciuperci, patogeni, Fusarium, Aspergillus, Penicillium, PCR.*

Introduction

Significant losses of maize yield worldwide are associated with fungal pathogens [1–3]. Maize pathogens from *Fusarium*, *Aspergillus* and *Penicillium* genera propagate on decaying plant residues, and conidia can infect plants both through roots or injuries caused by insects and abiotic factors during the whole period of vegetation [4–6]. These fungi present an exceptional threat for they comprise main pathogenic species that cause maize rots, as well as major producers of mycotoxins [7–9]. Two distinct diseases in maize are associated with *Fusarium*: Giberrella ear rot, or GER, caused by *F. graminearum* and *F. culmorum*; and Fusarium ear rot, or FER, caused mainly by *F. verticillioides* and *F. proliferatum* [10]. *Aspergillus* fungi can infect maize plants during vegetation; however they are more widely associated with contamination of kernels with mycotoxins rather than considered causal agents of major fungal diseases [11]. *Penicillium* fungi mostly cause kernel rot during storage [12]. Most of the fungal pathogens associated with corn diseases and yield spoilage are soil-borne, and soil itself represents a natural reservoir of plant infection with a wide specter of fungi. Therefore, a special attention should be focused on the phytosanitary state of the soil for assessing potential risks and diminishing adverse impact of fungal infection on maize cultivation.

The aim of this work was to evaluate the dynamics of several most important fungal maize pathogens from *Fusarium*, *Aspergillus* and *Penicillium* genera in soil under weather conditions of Republic of Moldova.

Material and methods

Samples of upper soil layer (≤10 cm) were collected on experimental cornfields of Institute of Genetics, Physiology and Plant Protection at the end of maize vegetation. Values of air temperature, relative air humi-

dity and quantity of precipitations were obtained from State hydrometeorology service, Chisinau [13]. A 1 g of dried soil was used for total DNA extraction using a validated protocol [14]. Pathogens' identification was performed via nested-PCR assay using a set of home-design primers to specific regions of fungal genome (Tab. 1) presented in GenBank database [15].

Table 1. Primers used for identification of some fungal pathogens.

Primer	Sequence 5'→3'	Species	Target gene
feqin1	CTGGGCTTCGGCAGGTCAA	*F. equiseti*	*tub2*
feqin4	GCGCGTGAGCTTGTTGTGTT		
fqeqin2	TCCCCAGAATCAATACGCTAACC		
fqeqin3	TCACTGGGTAACAAGGTCGAAGA		
fqin1	ACGTGGCGGGGTAATTTCAT	*F. incarnatum*	*tef1*
fqin2	GGAAACCAACCTTCTCGAACTTCT		
fspte1	CAGACTTGGCGGGGTAGTTTC	*F. sporotrichioides*	*tef1*
fspte4	GAGCGTCTGGTAGGCATGTT		
fqspte2	CTCTCATACGACGACTCGACAAG		
fqspte3	TGTGTGGGAAGGGCAAAAGC		
fcute1	CATCCCAACCCCGCCGATA	*F.culmorum*	*tef1*
fcute4	CGCGCCTAGGGAATGGTTTG		
fcute2	CGATACATGGCGGGGTAGTTT		
fcute3	ATGAGCCCCACCAGAAAAATTACG		
ftri8gr1	CTTCCGGTAATGTTTCTCGTCACT	*F. graminearum*	*TRI8*
ftri8gr4	CGCTGCTGAGGGTTTTACCAT		
fqtri8gr2	CTCGTCACTTCCTTGATGACACA		
fqtri8gr3	GGGGGCCGACATTCACTTC		
fufum6ve1	GCCTTTGTTTGGGGCCATGA	*F. verticillioides*	*FUM6*
fufum6ve4	CTGAGACCCTCGCCAGTTTTG		
fqfum6ve2	TCGCCCTTTGCACCATTGAC		
fqfum6ve3	AGCCTGCCGCTTGAACTTTG		
fprfum61	TCGGATTGTCACGCCTTTGT	*F. proliferatum*	*FUM6*
fprfum64	GTCCTTGGCGTTCAGCATTG		
fqprfum62	ATCGCCCTCTGCACGATAGA		
fqprfum63	TGGGAGGTTGCTCTGAGTGA		
afap1	CTTTGTTCGGTAGTGCCATCTTGA	*A. flavus*	*aflP*
afap4	GCCATAGCACATATTCTCCAACCT		
aqfap2	GTGTCGGGTGTGCCTATTTAACC		
aqfap3	AAGGCTTTCGGTCGGTTGATG		
apap1	TTGCTCGGTAGTGCCATGTT	*A. parasiticus*	*aflP*
apap4	GGCTTCCATAACACATATTCTCCAA		
aqpap2	CCGCGAAAGAACAAACAGAGA		
aqpap3	AACACATATTCTCCAACTTTCTTGCT		
acl1	TGATGGAAGATTCAGAGCCATACA	*A. clavatus*	*tub2*
acl4	CGTGAAGGTTTTGTCGAAGCA		
aqcl2	CGTGGATATGGGCTGACAGATTTA		
aqcl3	ATTTCCATCAGATCCATGCTCAGT		

aoch1	CAGGATCATCTTCGATACCTTAGGA	*A. ochraceus*	*tub2*
aoch4	GCCACCGGAAGCCTAGAAG		
aqoch2	ACCCATCGAAATCAGAGTCGAAAA		
aqoch3	CGTTTCGAATAGGCGAAAGGAGATA		
pcit1	CCTTGATGGCGATGGACAGT	*P.citrinum*	*tub2*
pcit4	CAGCACCGGATTGACCGAAA		
pcit2	CTACAACGGAACCTCCGATCTC		
pcit3	AGCACCGGATTGACCGAAAA		
pex1	AACGCCACTCTCTCCGTTCA	*P. expansum*	*tub2*
pex4	GGGTCAACTCGGGGACGTT		
pex2	CTCTCTCCGTTCACCAGCTT		
pex3	TCAACTCGGGGACGTTGAC		
pgr1	TGGATTACAGGCAAACCATTTCC	*P. griseofulvum*	*tub2*
pgr4	ACCGCTAGCCTAGATGATCAAA		
pqgr2	CCAGTGGATGGCATGTCTGAT		
pqgr3	TTCGGTTCCCAGTCGGCTAT		
pver1	ATTGACGGGTTCCTAACTTGGATT	*P. verrucosum*	*tub2*
pver4	ATGCACGTTTATTCCGGTTCCA		
pqver2	TCGATGGTGACGGACAGTAAGT		
pqver3	GGTTCCAGTCGTTGAACTCACAT		
pbrbt1	CCGCTATGGCTGGGTATCAAT	*P. brevicompactum*	*tub2*
pbrbt4	TAGCCTGGGCGGTCAAGAATA		
pbrbt2	TGCTAACTGGCTCAAAGGCAAA		
pbrbt3	TGCACAACCAGAGTTCTTCACA		
pchbt1	GTTGCTAACTGGATTACAGGCAAAC	*P. chrysogenum*	*tub2*
pchbt4	CACCGCTGGCCTAGATTGTC		
pqchbt2	TGATGGGGATTCTGGTGGATCA		
pchbt3	CCGCTGGCCTAGATTGTCAAA		

Analysis of soil microflora included qualitative analysis using nested-PCR and quantitative conventional PCR with Poisson distribution.

Nested-PCR included two subsequent amplification for 30 cycles each in a 25µl PCR-mix containing 66 mM Tris-HCl (pH 8.4), 16 mM $(NH_4)_2SO_4$, 2,5 mM MgCl*2*, 0,1 % Tween 20, 7 % glycerol, 100µl^{-1} BSA, 0,2 mM of each dNTPs, 1,25 U Taq DNA polymerase (Thermo Fisher Scientific), 5 pM of each primer and 10 ng of DNA. The first round included 3 minutes of preliminary denaturation at 95°C, followed by 30 cycles which included 30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C and 30 seconds of elongation at 72°C. The second round included 30 cycles which comprised 30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C and 30 seconds of elongation at 72°C. Both rounds ended with 7 minutes of terminal elongation at 72°C. All samples that showed positive amplification with certain primer pairs were used for further quantitative analysis.

Pathogen quantification was performed using conventional PCR with Poisson distribution assay: a total of 10 ng of extracted DNA was divided in *n* reaction samples containing PCR-mix, each was amplified as described for round I nested-PCR. Copy number was calculated using the following formula:

m=-n* ln(E)

where m – copy number of target-sequence per sample, n – number of particles, E – probability of null particle (no amplification).

The products of amplification were separated in 1,5% agarose gel electrophoresis (6V/cm) in a 1xTBE migration buffer (pH 8.0) with ethidium bromide, viewed in the UV (302 nm) and photographed.

Means' comparison was performed using one-way ANOVA test ($p<.05$). Correlation between fungi quantities in soil and mean values of abiotic factors (air temperature, relative humidity and quantity of precipitations) was evaluated using Pearson's correlation coefficient.

Results and discussions

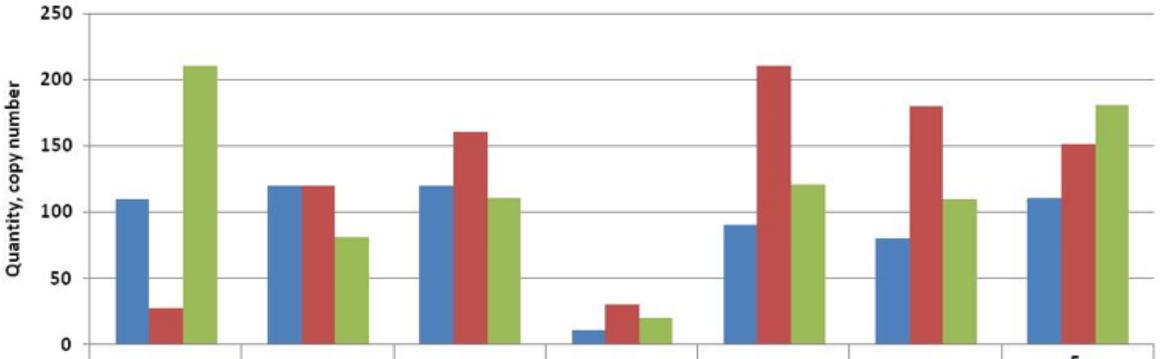
The development and propagation of fungal pathogens depend on abiotic environmental factors, among which temperature and air humidity are limiting. The values of average daily temperatures differed significantly ($p<.0001$) only in March and April before corn sowing, during other months the difference in the values of this parameter between years was insignificant (Tab. 2). The values of relative air humidity in the spring-summer period varied more strongly between years, and May-July 2022 and July-August 2020 can be considered the most unfavorable for the development of fungal pathogens, when the values of this parameter did not reach 55%. The least amount of precipitation was recorded in April and August of 2020 and May-June of 2022.

Table 2. Values of abiotic factors, critical for fungi's propagation in spring-summer season during 2020-2022 period of monitoring.

	Average air temperature (°C)					
	March	**April**	**May**	**June**	**July**	**August**
2020	7.8	11.3	13.9	21.3	23.2	23.5
2021	3.4	8	14.8	19.7	23.5	21.3
2022	3.2	10.2	16.5	21.9	23.3	23.4
	Average relative air humidity (%)					
	March	**April**	**May**	**June**	**July**	**August**
2020	57	35	63	64	53	47
2021	67	64	68	71	64	66
2022	55	60	49	53	48	62
	Sum of precipitations (mm)					
	March	**April**	**May**	**June**	**July**	**August**
2020	20	4.4	66	86	85	4.5
2021	36	38	104	87	116	112
2022	11	70	21	6.7	82	82

Soil fungal microbiota was represented by *Fusarium*, *Aspergillus* and *Penicillium* genera. Seven *Fusarium* species were identified, *F. graminearum*, *F. verticillioides* and *F. proliferatum* being main causal agents of major maize diseases Gibberella ear rot and Fusarium ear rot (Fig.1). All three species are mycotoxigenic and are able to produce DON (F. graminearum) and fumonisins (F. verticillioides, F. proliferatum) [9]. Quantitative analysis using conventional PCR with application of the Poisson distribution demonstrated that among *Fusarium* pathogenic species the most abundant in the soil was *F. graminearum*, and the lowest amounts were calculated for *F. culmorum*. Although other three species, *F. equiseti*, *F. incarnatum* and *F. sporotrichioides*, are able to infect maize, they are considered minor pathogens that maize induce stalk and kernel rots usually with other major pathogens [10]. For most of the species the lowest quantity was calculated in 2020, when climate conditions were least favorable for fungal life cycle. It was observed a significant influence of environmental conditions at $p<.05$ on the quantity of *F. graminearum* [$F(2,15)=4.61$, $p=.027$], *F. equiseti* [$F(2,15)=5.26$, $p=.019$], *F. incarnatum* [$F(2,15)=8.18$, $p=.003$]. For all three species there was a statistically significant difference in pathogen's quantity in soil between years 2020 and 2021.

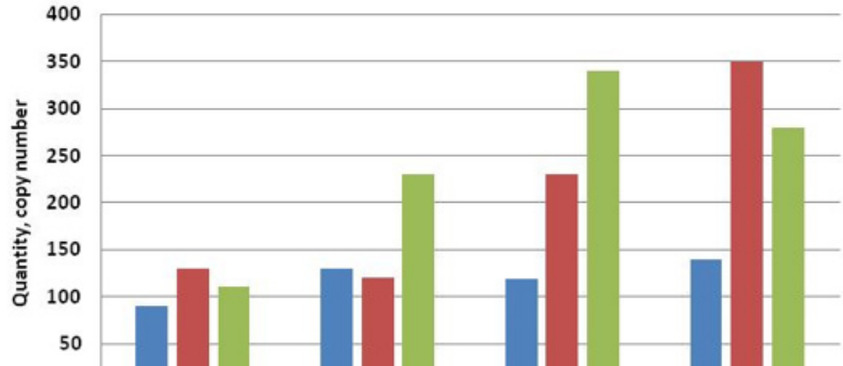
Fig. 1. Mean Fusarium spp. quantities in soil samples gathered from experimental cornfields.



	F. graminearum	F. verticillioides	F. proliferatum	F. culmorum	F. equiseti	F. incarnatum	F. sporotrichioides
2020	110.07	120.15	120.14	10.61	90.86	80.48	110.32
2021	27.57	120.15	160.82	30.69	210.27	180.1	150.84
2022	210.74	80.98	110.48	20.16	120.4	110.2	180.82

Results of PCR analysis showed that *Aspegillus* was the most abundant fungal genus in soil. The highest quantities were calculated for *A. clavatus* and *A. ochraceus*, main aflatoxigenic [11] fungi *A. flavus* and *A. parasiticus* were present in smaller quantities (Fig.2). Conditions of the year didn't affect significantly accumulation of *A. flavus*, *A. parasiticus* and *A. ochraceus* in soil. Only quantities of *A. clavatus* differed significantly under weather conditions of the year [F(2,15)=5.75, p=.014]. Significant difference in fungus's quantity was observed between years 2021-2022. During the first year of monitoring this difference was not significant.

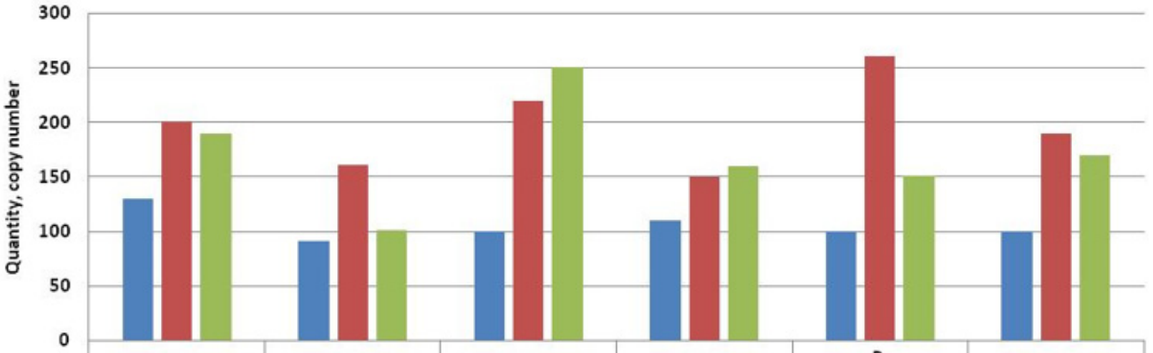
Fig. 2. Mean Aspergillus spp. quantities in soil samples gathered from experimental cornfields.



	A. parasiticus	A. flavus	A. clavatus	A. ochraceus
2020	90.48	130.58	120.09	140.54
2021	130.84	120.32	230.29	351
2022	110.74	230.29	341	280.25

Overall *Penicillium spp*. quantity in soil was higher compared to *Fusarium* fungi, the lowest quantities of *Penicillium* fungi in soil were observed in 2021 (Fig.3). PCR analysis revealed that the most prevalent was *P. griseofulvum* and *P. brevicompactum*. However, *P. citrinum* and *P. verrucosum* were less abundant. Overall impact of weather conditions of the year on the fungi's accumulation in soil was not very prominent. Year of vegetation affected significantly the quantities of *P. chrysogenum* [F(2,15)=3.85, p=.045], statistically differed the values between years 2020 and 2021. Also, quantities of *P. expansum* was affected by conditions of the year of vegetation [F(2,15)=8.98, p=.003] and significant difference in fungus' quantities was observed between years 2020-2021 and 2020-2022. Weather conditions did not affect significantly the accumulation of other *Penicillium* species in soil.

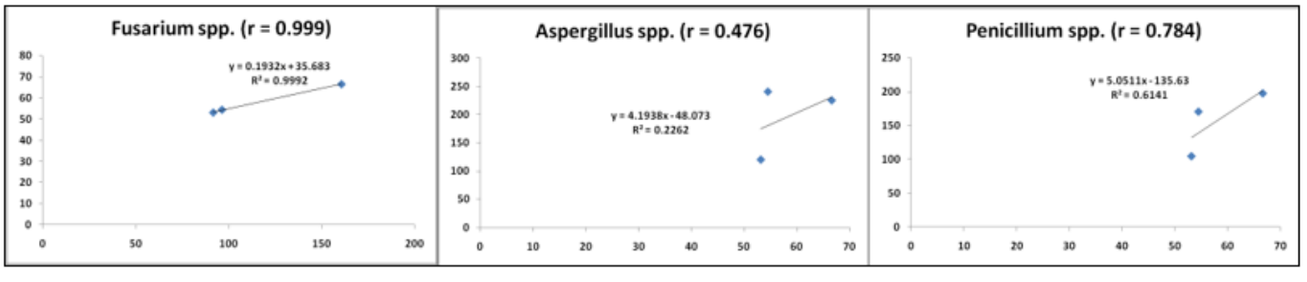
Fig. 3. Mean Penicillium spp. quantities in soil samples gathered from experimental cornfields.



	P. chrysogenum	P. citrinum	P. griseofulvum	P. verrucosum	P. brevicompactum	P. expansum
2020	130.32	90.71	100.48	110.13	100.05	100.09
2021	200.72	160.76	220.46	150.43	260.82	190.49
2022	190.1	100.89	250.89	160.49	151	170.3

A positive correlation between mean values of *Fusarium spp*., *Aspergillus spp*. and *Penicillium spp*. quantities in soil and mean values of relative humidity was observed (Fig.4). For *Fusarium spp*. correlation between fungi's quantity and values of the factor was very strong positive, while for *Penicillium spp*. correlation was strong positive and for *Aspergillus spp*. it was already moderate positive. Although air humidity is a restrictive factor for fungi's propagation, its impact can be diminished by the accessibility of moisture, and therefore uneven distribution of rainfall during the season of vegetation might affect the impact of air humidity on fungi's accumulation in soil. Difference in Pearson correlation coefficient for *Fusarium spp*., *Aspergillus spp*. and *Penicillium spp*. also might be explained by the additional sources of soil infestation with different fungi – *Aspergillus spp*. and *Penicillium spp*. are considerate „depositary fungi", therefore, maize seed material itself can serve as source of soil contamination with respective pathogens.

Fig. 4. Correlation between mean values of relative air humidity during spring-summer season (2020-2022) and quantities of Fusarium spp., Aspergillus spp. and Penicillium spp. in soil.



On the contrary, correlation between fungi's quantity in soil and air temperatures was negative (Fig. 5). A very strong negative correlation between the studied parameters was observed for *Fusarium spp*. and *Penicillium spp*., while for *Aspergillus spp*. there was a moderate negative correlation. *Aspergillus spp*. are more specific for southern regions and are able to propagate under higher mean temperatures compared to *Fusarium* genus [1]. The later comprises species that need different intervals of optimum air temperatures for propagation, for some of them lower temperatures are essential and they are more specific for temperate areas, like *F. graminearum* [9]. Summer heat affects negatively some maize genotype, susceptible to high air temperatures, and withered plants become substrate for propagation of saprotrophic fungi, weakening the negative correlation between quantities of *Aspergillus spp*. and *Penicillium spp*. in soil and values of relative air humidity during maize vegetation.

Generally, correlation between quantities of precipitations during the period of maize vegetation and quantities of precipitation showed a positive trend (Fig.6). For *Fusarium spp*. this parameter was strong positive, while for *Aspergillus spp*. it was weak. Therefore, quantity of *Aspergillus* fungi in soil was less influenced by quantities of precipitations compared to *Penicillium* and *Fusarium* fungi.

Fig. 5. Correlation between mean values of air temperature during spring-summer season (2020-2022) and quantities of Fusarium spp., Aspergillus spp. and Penicillium spp. in soil.

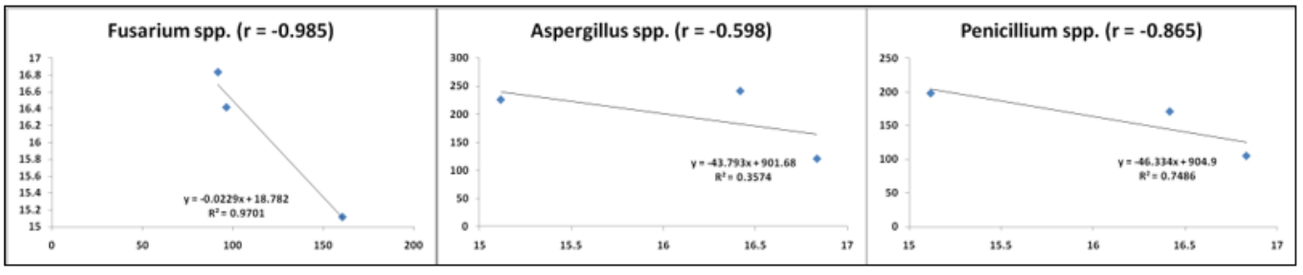
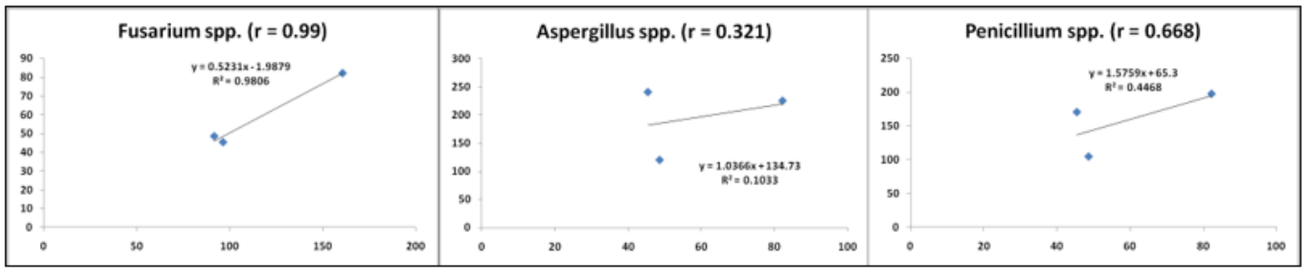


Fig. 6. Correlation between quantities of precipitations during spring-summer season (2020-2022) and quantities of Fusarium spp., Aspergillus spp. and Penicillium spp. in soil.



Thus, major and minor causal agents of FER and GER as well as opportunistic pathogens like *Aspergillus spp*. and *Penicillium spp*. that provoke grain spoilage during storage were identified in soil during 3-year period of monitoring. Many of these species are able to produce fumonisins, aflatoxins, trichotecenes, ochratoxin A, patulin and several other mycotoxins, which have a serious adverse impact on human health, livestock's productivity and quality of maize grain during storage in Gene bank.

Conclusions

Soil from experimental cornfields was contaminated with several pathogenic species of fungi that cause major diseases in maize (FER and GER) and a wide specter of fungi that induce kernel spoilage during storage. These fungi were identified in soil samples during the whole period of monitoring, and weather conditions of the year of vegetation had an uneven impact on their accumulation in soil resulting from different optimum values of abiotic factor for different fungi's propagation and action of additional external factors. Positive correlation was observed between quantities of fungi and values of relative air humidity. It was revealed that propagation of *Fusarium spp.* was the most affected by abiotic factors compared to the species from two other fungal genera.

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