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GRAJDIERU CRISTINA

MOLECULAR IDENTIFICATION OF MAIZE (*ZEA MAYS* L.) GENOTYPES RESISTANT TO SOME PATHOGENIC FUNGI

162. 01. Plant genetics

Summary of PhD thesis in biology

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The thesis was developed within the Laboratory of Molecular Genetics of Institute of Genetics, Physiology and Plant Protection of the Moldova State Universtity.

Scientific advisor **–**

TUMANOVA Lidia	PhD in chemistry, research lecturer, Moldova State University

Composition of the Doctorate Commission**:**

DUCA Maria	Dr. habil in biology, university professor, academician, Moldova State University – ***president***
TUMANOVA Lidia	PhD in chemistry, research lecturer, Moldova State University – ***scientific advisor***
LUPAȘCU Galina	Dr. habil in biology, research professor, Moldova State University – ***reviewer***
COMAROVA Galina	PhD in biology, associate professor, Technical University of Moldova – ***reviewer***
CEPOI Liliana	PhD in biology, research lecturer, Technical University of Moldova – ***reviewer***

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The doctoral thesis and the summary can be consulted at the National Library of the Republic of Moldova, Central Scientific Library "Andrei Lupan" (Institute), Central Library of the Moldova State University (MD 2009, Chisinau, 60 Alexei Mateevici str.), on ANACEC website (http://www.cnaa.md) and MSU website (http://www.usm.md).

The summary was sent on the 26.03.2024.

President of the Doctorate Commission Dr. habil in biology, university professor, academician		**DUCA Maria**
Scientific advisor PhD in chemistry, research lecturer		**TUMANOVA Lidia**
Author:		**GRAJDIERU Cristina**

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CONCEPTUAL MILESTONES OF THE RESEARCH

Actuality of the subject. Maize is one of the three cereal crops that satisfy human food needs worldwide. Considering its valuable nutritional properties and versatile field of use, maize remains the subject of many breeding programs that mainly focus on obtaining genotypes comprising high productivity and resistance to biotic and abiotic stress [1-3]. Maize cultivation faces several obstacles that reduce the quantity and quality of the yield. Fungal diseases are mentioned among them, especially those caused by fungi of the genera *Fusarium*, *Aspergillus* and *Penicillium*. These three genera include both highly pathogenic and aggressive species (*F. graminearum, F. verticillioides*), which cause systemic diseases in plants during the growing season, and so-called minor pathogens – fungi, which by themselves rarely induce systemic infections and are associated with grain damage during storage [4-6]. These three genera comprise most species producing mycotoxins – secondary metabolites with toxic effect in animals and humans. Acute toxicity, immunosuppressive effect, nephrotoxicity, induction of malignant tumor formation, neurotoxicity are named among the adverse physiological effects of these compounds [7-9]. Mycotoxins are a serious economic problem and maize is one of the three crops most commonly affected by mycotoxins. Modern agriculture offers various corn cultivation technologies that lessen the negative impact of pathogenic fungi on this crop, including crop rotation, effective pesticides, physiologically active compositions, biological control agents and many more. However, there are no signs that the area of *Fusarium*, *Aspergillus* and *Penicillium* invasion is decreasing, while mycotoxins remain an emerging hazard worldwide. Thus, there is a need to develop procedures to reduce the risks associated with the infection of corn with fungi from the genera *Fusarium*, *Aspergillus*, *Penicillium* and the contamination of production with mycotoxins.

Current research achievements. From the point of view of conserving biodiversity, reducing environmental contamination with pesticides, as well as obtaining ecological food products, it is reasonable to cultivate maize genotypes resistant to fungi, including toxigenic ones. However, to date, the *R* genes that determine the absolute resistance of maize to *Fusarium*, *Aspergillus* and *Penicillium* fungi have not been discovered, and the agricultural market does not have maize genotypes with absolute resistance to fungi of these genera [10]. Different infection rates of maize with named fungi are associated with a large number of QTL. Each of these QTL has a non-significant individual contribution to the formation of resistance, they are unevenly disseminated among geographic maize populations, act in ensembles, are strongly influenced by environmental factors, and have complex inheritance [11, 12]. All this prevents the identification of resistant genotypes based on molecular screening alone. As a result, the selection of plants of

interest can be carried out by field trials using reliable and robust methods for assessing the degree of host resistance to fungi. Based on the fact that the mechanisms of resistance to phytopathogens (induced or constitutive) are aimed at preventing their penetration into the host plant or suppressing their growth and development, it can be concluded about the degree of sensitivity of maize genotypes to fungal infections using the rates of infection and/or amount of pathogens in plant tissues. This procedure assumes the precise identification of the harmful species and the availability of a quantitative method that allows estimating the content of pathogens in plant organs. Its achievement is carried out by conventional methods, which include the evaluation of plant disease symptoms and the identification of fungal pathogens based on morphological and biochemical criteria. However, their application is hampered by some difficulties. Many phytopathogens cause similar symptoms, and their manifestation may differ depending on environmental factors, the vegetation phase and the physiological state of the plant; in the initial stages of the infectious process, the disease often does not show distinct symptoms. Microbiological and biochemical methods require special rooms and apparatus for the cultivation of pathogens, which implies taking additional safety measures to prevent contamination, and are lasting; some species of phytopathogens have similar morphological and biochemical characters and do not sporulate on artificial media. Furthermore, mycotoxin-producing fungal strains cannot be discriminated from non-toxigenic strains by morphological traits alone. The difficulties mentioned above are overcome by using molecular methods, mainly PCR. The PCR method is based on the use of genomic sequences as molecular markers for the identification of fungi. Currently, the GenBank database provides a significant amount of genomic sequences, which allow the identification of fungi at different taxonomic levels. Therefore, PCR-based tests are effective as diagnostic tools because they use genomic traits that are stable and heritable, and in many cases do not require the cultivation of fungi on artificial media. On the other hand, the rapid evolution of fungi constantly requires the analysis of genomic sequences, identification of markers, development of primers and the optimization of PCR protocols for the molecular diagnosis of pathogenic fungi and the estimation of the degree of susceptibility of maize to causal agents of infectious diseases and accumulation of mycotoxins in tissues.

Aim: development of rapid and accurate methods for assessing the susceptibility of maize to fungal pathogens in order to select germplasm valuable as material for maize breeding programs based on resistance to fungi of the genera *Fusarium*, *Aspergillus*, *Penicillium*.

Main objectives:

1) testing *de novo* designed primers based on housekeeping gene sequences and gene clusters in the fungal genome encoding enzymes involved in the biosynthetic pathways of different classes of mycotoxins;
2) development and optimization of end-point PCR and real-time PCR protocols for the qualitative, semi-quantitative and quantitative analysis of fungi in the organs of maize plants; analysis of the phytosanitary status of fields and maize germplasm stored in the Gene bank of the IGPPP;
3) identification of genotypes of interest from the active collection of the IGPPP in order to use them in national programs to improve maize resistance to fungal diseases.

Metodology. Qualitative and quantitative analyzes of fungi in the biological material were performed using nested-PCR, real-time PCR methods. Qualitative and quantitative analyzes of the main classes of mycotoxins were performed using commercial ELISA kits (Elabscience).

Research hypothesis. Combating systemic diseases in plants is based on the action of constitutive and induced resistance factors, which prevent the penetration of the pathogen and its propagation in the host plant. Thus, the analysis of the frequency and dynamics of the accumulation of fungi in the organs of different varieties and lines of corn under the uniform action of environmental factors allows concluding about the degree of resistance and susceptibility of the analyzed corn genotypes.

Scientific quality, innovation potential and value of results. The current study concept is based on relevant scientific data on the monitoring of pathogens in agricultural crops using molecular methods. The innovative potential includes the attempt to evaluate maize germplasm stored in the IGPPP Gene Bank for the identification of resistance donors to fungi from the genera *Fusarium*, *Aspergillus* and *Penicillium* using PCR-based assays. The objectives achieved will contribute to the broadening of scientific and technological knowledge regarding the dissemination and evaluation of pathogenic fungi in cornfields and storage facilities. PCR primers and protocols can be recommended to national phytosanitary divisions and food safety control centers for the assessment of emerging risks of contamination of crops and grain-derived products with pathogenic and toxigenic fungi, as well as scientific subdivisions specialized in the fields of microbiology, ecology and phytopathology.

Structure and volume of the thesis. The thesis consists of introduction, 4 chapters, general conclusions and recommendations, bibliography of 236 titles, 116 pages of body text, 48 figures, 14 tables, 2 appendices, statement of responsibility, candidate's CV.

Dissemination of results. The research results were presented in 20 scientific papers, of which three were published in journals indexed in databases accepted by ANACEC: Evaluarea

rezistenței a plantelor de porumb la speciile de *Fusarium* prin metoda PCR. In: *Studia Universitatis Moldaviae (Seria Ştiinţe Reale şi ale Naturii)*. 2023, nr.1 (171), pp. 133-138; Dynamics of maize pathogens from *Fusarium*, *Aspergillus* and *Penicillium* genera in soil under weather conditions of Republic of Moldova. In: *Studia Universitatis Moldaviae (Seria Ştiinţe Reale şi ale Naturii)*. 2023, nr.1 (171), pp. 106-112; Evaluarea liniilor consangvinizate de porumb în baza rezistenței la fungi toxigenici din genurile *Fusarium* și *Aspergillus*. In: *Studia Universitatis Moldaviae (Seria Ştiinţe Reale şi ale Naturii)*. 2022, nr.1 (151), pp. 35-41. Two articles were published in journals from the National Register of Scientific Journals (category B): Monitoring of *Penicillium* infection during eggplan tontogenesis. In: *Buletinul Academiei de Ştiinţe a Moldovei. Ştiinţele vieţii*. 2022, nr.3 (343), pp. 48-53; Poisson distribution-based conventional PCR protocol for quantification of pathogenic fungi in maize. In: *Buletinul Academiei de Ştiinţe a Moldovei. Ştiinţele vieţii*. 2021, nr.2 (344), pp. 92-97. Two articles were published in international conference proceedings: Identificarea moleculară a fitopatogenilor a porumbului la faza generativă de dezvoltare a plantelor. În: materiale conferinței internaționale ”Тенденции развития агрофизики: от актуальных проблем земледелия и растениеводства к технологиям будущего” (ediția a IV-a), 13-15 septembrie 2023, Sankt-Petersburg. Sankt-Petersburg: ARI, 2023, pp. 39-44; Quantitation of toxigenic Aspergillus flavus strains in maize seed material via conventional PCR. In: Materialele conferinței internaționale ” Тенденции развития агрофизики: от актуальных проблем земледелия и растениеводства к технологиям будущего”(ediția a III-a), 14-15 septembrie 2021, Sankt-Petersburg. Sankt-Petersburg: ARI, 2021, pp.255-258. Experimental data were presented in oral communications at scientific events, 5 of which were international: Simpozionul științific internațional „Protecția plantelor – realizări şi perspective”, 2-3 octombrie 2023, Chișinău; Conferința internațională ”Тенденции развития агрофизики: от актуальных проблем земледелия и растениеводства к технологиям будущего” (ediția IV), 13-15 septembrie 2023, Sankt-Petersburg; Simpozionul internațional ”Advanced Biotechnologies – Achievements and Prospects” (VIth Edition), 3-4 octombrie 2022, Chișinău; Conferința internațională ”Тенденции развития агрофизики: от актуальных проблем земледелия и растениеводства к технологиям будущего”, (ediția III), 14-15 septembrie 2021, Sankt-Petersburg; Simpozionul internațional ”Protecția plantelor – realizări și perspective”, 27-28 octombrie 2020, Chișinău.

Implementing scientific results. The molecular diagnostic procedures exposed in the thesis were used to solve some applicative problems: Evaluation of the phytosanitary state of apple orchards in the Republic of Moldova and the effectiveness of several fruit storage conditions in order to reduce their damage caused by microorganisms (bilateral project STCU-AŞM # 6225);

complex analysis of the accumulation of mycotoxins in foods during storage (moldo-belarus project 19.80013.51.07.10A/BL); identification of tomato genotypes resistant to phytoplasma (bilateral project STCU-AŞM #6378); optimization of procedures for reducing wine contamination with microorganisms and their metabolites (TUBITAK project 23.80013.5107.4TR). The results of the scientific investigations carried out in the thesis were implemented in the practical works of the discipline ” Research techniques in molecular biology”, cycle II at the Department of Biology and Ecology, Faculty of Biology and Geosciences of MSU (implementation act nr. 507i from 08.06.2023)..

THESIS CONTENT

The **introduction** reflects the importance of the topic addressed the purpose and objectives, the theoretical and practical relevance of the results obtained the synthesis of the chapters and the dissemination of the results.

Chapter I. THE MAIN FUNGAL DISEASES OF CORN: CAUSAL AGENTS, PATHOGENESIS AND EVALUATION METHODS OF MAIZE RESISTANCE TO FUSARIOSIS, ASPERGILLOSIS AND PENICILLOSIS

The chapter summarizes the information on the ecology and economic impact of fungi of the *Fusarium*, *Aspergillus* and *Penicillium* genera on maize. The main pathogens that induce systemic diseases in corn during the growing season and the fungal species that most often cause grain damage during storage are described. The main classes of mycotoxins produced by some species of filamentous fungi belonging to the mentioned genera are named together with their physiological effect, the legislative bases that regulate the permissible concentrations of mycotoxins in food products. The molecular basis of maize resistance to the main causal agents of fungal diseases and the accumulation of classes of mycotoxins are described.

Chapter II. MATERIALS AND METHODS

Methods of sampling and analysis of DNA samples extracted from soil and maize organs are described. Maize genotypes from the IGPPP active collection were used in the study: CP137, MK01, Ku123, B73, CP148, MAN2281, MAN2459, MAN2461, MAN2451, MAN2308, MAN2526, MAN2425, MAN2452, MAN2414, MAN2413, MAN2424, MAN2448, MAN2488, MAN2493, MAN2491, MAN2483, MAN2470, MAN2466, MAN2463. The plants were grown on the experimental plots of the IGPPP in the period 2019-2022 with compliance with the agrotechnical procedures for the given crop. Soil was collected from experimental plots planted with maize at the end of the growing season. DNA extraction was performed using the method developed based on validated nucleic acid extraction protocols using SDS and CTAB buffer solutions [13-15The identification of fungi was carried out by the multiplex-PCR, nested-PCR,

real-time PCR method with a set of specific primers developed de novo in the Plant Genetics laboratory, IGPPP. The identification and quantification of mycotoxins (aflatoxins, fumonisins, zearalone, deoxynivalenol, T-2 toxin) was carried out using the ELISA method with commercial kits (Elabscience). The analysis of the genetic polymorphism of the maize genotypes was carried out with the primers derived from the sequences of the mobile elements of the plant genome, their effectiveness was evaluated with the help of the main descriptors: PIC, resolution power R, effective multiplexing rate E, marker index MI, discrimination power D. The statistical analysis of the obtained data was carried out based on the Levene, Shapiro-Wilk, ANOVA, Pearson coefficient tests. The clustering was performed by the UPGMA algorithm, the optimal coefficient was chosen by evaluating the cophenetic matrices. GelAnalyzer, Microsoft Office 2013, POPGENE ver. 1.32, GIMP ver. 2.10.24, Primer3 ver. 4.1.0 software were use for organizing the data and statistical analysis.

Chapter III. PRIMER TESTING AND OPTIMIZATION OF PCR PROTOCOLS FOR THE QUALITATIVE AND QUANTITATIVE ANALYSIS OF PATHOGENIC FUNGI IN PLANT AND SOIL SAMPLES

The DNA extraction method proposed in the given study allowed obtaining total DNA with a yield of about 80-100 ng/µl and high enough purity to perform nested-PCR and qPCR analyzes (Fig.3.1). At the same time, highly toxic reagents (phenol) and expensive enzymes (proteinase K) are not used in the extraction process, and the separation of proteins is done by coagulation with the help of CH_3COONH_4 7.5 M solution at low temperatures.

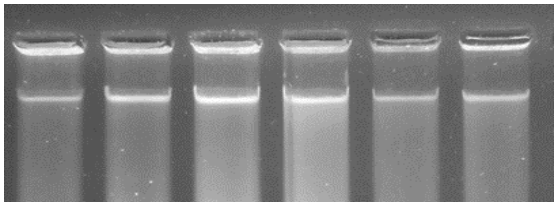

Fig.3.1. Electrophorogram of total DNA samples extracted from maize kernels by the combined SDS-CTAB method

The evaluation of the specificity of the primers was performed by BLAST analysis and experimental amplifications with the optimization of the parameters of alignment, elongation, the number of cycles and the amount of template DNA. Obtaining the amplicon of the predicted size and the lack of synthesis of non-specific products define the successful nested-PCR reaction (Fig.3.2). The primers demonstrated high specificity in the alignment range 57-60°C, the nested-PCR protocol included 30 cycles in each round and approx. 10 ng of template DNA.

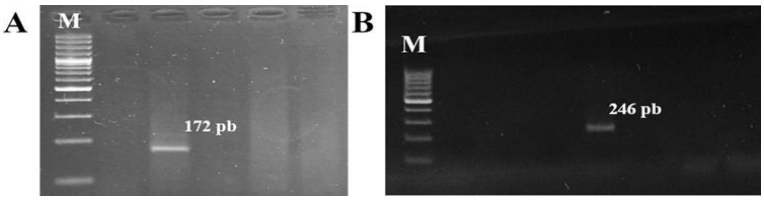

Fig. 3.2. Electrophorogram of amplicons obtained from PCR with primers pchbt1-pchbt4 and pchbt2-pchbt3 (A), pch2-pch3 (B). M – molecular marker GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific

Optimization of the qPCR protocol was performed based on the analysis of amplification and dissociation curves (Fig.3.3). The amplification curve represents the dependence of relative fluorescence values (RFU) on the number of PCR cycles. The accumulation of the fluorescent signal determines the positive reaction. The absence of the DNA of interest in the reaction mix results in the RFU values not exceeding the background values and the graph showing a straight line. Based on the dissociation curve, the dissociative properties of the amplicon were analyzed and the temperature at which the single-stranded DNA reassociates with the complementary strand and returns to its double-stranded form was determined. The analysis of the dissociation curve gave the information about the amount, length and structure of the amplicon obtained. It is observed that the amplification curves for the mqtri11sp7-mqtri11sp8 primer pair specific to the *TRI11* gene (*Fusarium spp*.) associated with DON and T-2 synthesis in the samples without target DNA represent a straight line, while the amplification curve for the MAN2414 sample containing copies of the *TRI11* gene defines an exponential increase in fluorescence, i.e. of the amplified fragment. The dissociation curve shows a single clearly defined peak with a melting point of 79°C, indicating the homogeneity of the amplified fragment and the specificity of the tested primer pair. The late transition (after cycle 30) to the exponential phase and exceeding the relative fluorescence threshold values proves the small amount of the gene of interest in the analyzed sample.

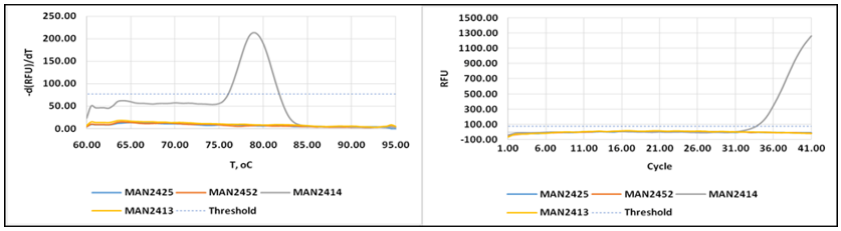

Fig.3.3. Dissociation (left) and amplification (right) curves of fragments obtained in real-time PCR using the mqtri11sp7-mqtri11sp8 primer pair at the TRI11 gene (dashed line – fluorescence threshold values)

The efficiency of the qPCR reaction was evaluated based on a single reaction. The analysis involves evaluating the dynamics of the change in RFU values during amplification using different mathematical approaches. In the current work, the following procedure is used:

- The logarithms of the RFU values in a base 2 are derived.

- The values obtained in the exponential section of the amplification curve are selected. Terminal values are excluded because in the transition to the plateau section the reaction efficiency naturally decreases due to depletion of the reaction mixture and inhibition with the final product, and close to the threshold values, the actual RFU values are disturbed by background fluorescence. Thus, 4-6 intermediate values are selected.
- The selected values are plotted along the respective cycle values, the regression curve is generated. The coefficient *a* from the obtained equation $y = ax + b$ denotes the efficiency of the reaction.

Efficacy ranged from 96-108% and therefore fits the desired amplification range of 90-110%. The data obtained demonstrate that the Taq polymerase works at near maximum capacity and the interference of secondary factors on the accumulation of the final product is insignificant.

Validity of using Cq, normalized Cq and nested-PCR values based on serial dilutions for semi-quantitative analysis of pathogens in the sample and estimation of susceptibility of maize genotypes to fungal infections has been demonstrated [16]. The method for quantifying pathogenic fungi based on the conventional PCR reaction, which approaches the principle of the digital-droplet-PCR method, was developed and tested. The principle of the technology consists in partitioning the total DNA into a number of individual samples, which are analyzed by PCR, positive signals are counted and then the number of sequences of interest in the particle or sample is deduced. The probability that the sample will contain *k* copies of the target sequence is governed by the binomial and Poisson distribution. If the number of particles n is large enough and the distribution is randomized, the probability of the target sequence appearing in the particle can be deduced from the 1/n relationship. When *n* is large enough (*n*=30 and more) and the probability 1/n is small, the binomial distribution can be approximated by the Poisson distribution:

$$m = -n * \ln(E)$$

where *m* represents the number of the target sequence in the sample, *n* – the number of partitions in the reaction series, *(E)* – the probability of null partitions.

Total DNA samples extracted from the grains of CP137 and CP148 genotypes and primers for the identification of *Aspergillus spp* fungi were used for the analysis. For the quantification of the sequences of interest, the total DNA solution was evenly distributed in 30 reaction mixes in which the amplification was carried out by conventional PCR. After amplification, negative samples (absence of the amplicon of interest) were counted and the copy number of the sequence of interest was calculated. The obtained results were compared with the Cq values obtained by qPCR calculated for the same samples with the same primers and represented graphically on the Bland-Altman diagram (Fig.3.4).

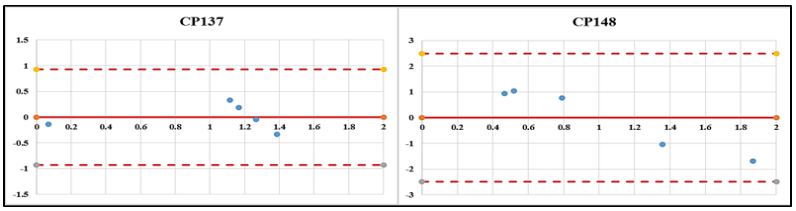

Fig.3.4. Bland-Altman plot of the number of copies of genes involved in the synthesis of aflatoxins in *Aspergillus spp*. for genotypes CP137 and CP148 obtained from end-point PCR with the application of Poisson distribution and Cq values normalized to the 18S sequence

Thus, conventional PCR with the application of the Poisson distribution can be used in certain cases as an alternative to the qPCR reaction. The advantages of this procedure in that the given protocol does not require special equipment and/or additional reagents and can be performed on a conventional PCR amplifier. However, the given procedure requires the performance of a large number of reactions for the analysis of a single sample, and the reduction of the number of partitions in the amplification series leads to the increase of the error and the miscalculation of the number of copies in the sample. Thus, this method is not effective when it is necessary to analyze a large number of samples.

Chapter IV. THE IMPACT OF ENVIRONMENTAL FACTORS AND GENOTYPE ON THE RATE OF ACCUMULATION OF FUNGAL PATHOGENS IN MAIZE PLANTS

Phytosanitary status of cornfields. Monitoring the phytosanitary status of cornfields is necessary for forecasting the spread of fungal infection and taking preventive measures before sowing and storage. The propagation of fungi is significantly influenced by the abiotic factors of the environment, air temperature, amount of precipitation and air humidity being limiting for the development and multiplication of microorganisms. Air temperature values during the monitoring period (2020-2022) varied uniformly and no significant influence of the year on the respective parameter was observed. Unlike the temperature, the values of the relative humidity of the air varied more pronounced between years. Based on this parameter, the most unfavorable months for the development of fungi were May-July 2022 and August 2020. The lowest amounts of precipitation were recorded in the months of April and August 2020, May and June 2022.

F. graminearum, F. verticillioides, F. proliferatum, F. culmorum, F. equiseti, F. incarnatum, F. sporotrichioides, A. parasiticus, A. flavus, A. ochraceus, A. clavatus, P. chrysogenum, P. expansum, P. citrinum, P. griseofulvum, P. verrucosum, P. brevicompactum were identified in the surface soil layer [19]. Thus, species associated with infectious diseases and damage to maize production were present in the soil. Most of the microorganisms detected were represented by toxigenic fungi, identified based on the genomic sequences involved in the

biosynthetic pathways of the main classes of mycotoxins. The amount of fungi in the analyzed samples varied depending on the species and the year of collection. The quantitative analysis demonstrated that the most abundant microorganisms belonged to the genus *Aspergillus*, followed by *Penicillium spp*. *Fusarium* species were present in smaller amounts (Fig.4.1). The conditions of the year unevenly affected the dynamics of fungi in the soil; no correlation was established between the pathogenicity of the fungi and their accumulation in the soil.

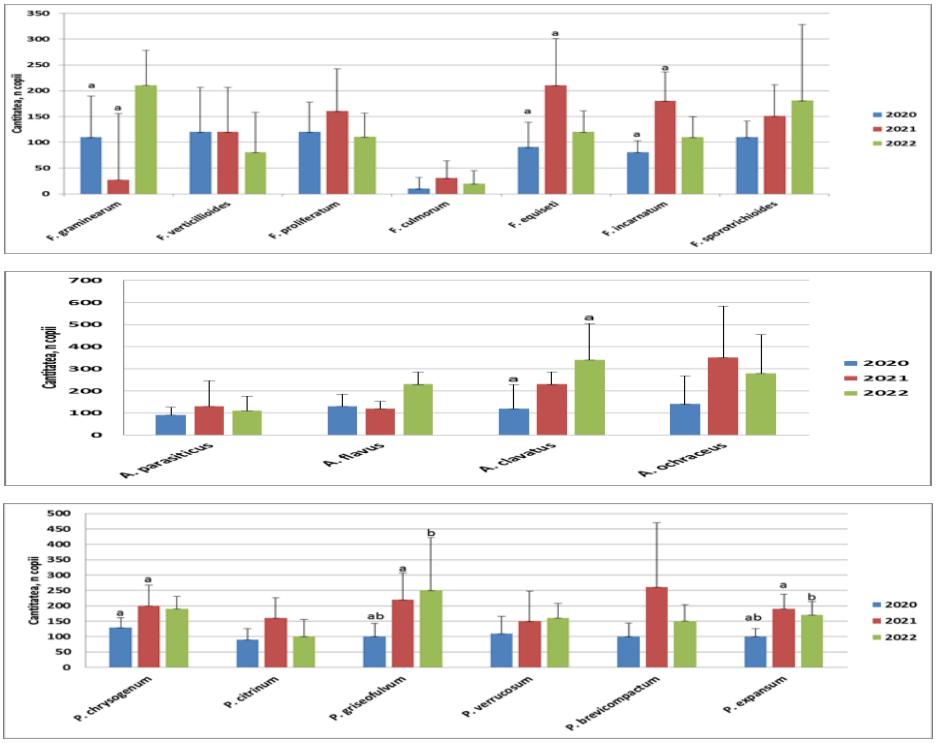

Fig.4.1. Average amounts of fungi in the soil (number of copies per 100 mg of soil). Letters indicate values that differed significantly at p<.05

In contrast to soil, in plant material samples *F. culmorum* was absent in both leaves and grains throughout the entire growing season. Environmental factors more significantly influenced the amount of fungi in plant organs, especially in leaves that are more strongly exposed to environmental factors compared to grains. The most abundant *Fusarium* species in leaves were *F. verticillioides* and *F. proliferatum*, *F. graminearum* was identified less frequently and in smaller quantities. Among minor pathogens the most frequent and abundant was *F. equiseti* – zearalenone producer, and the frequency and average amount of *F. incarnatum* and *F. sporotrichioides* was lower (Fig.4.2). In grains, the amount of *F. graminearum* and *F. verticillioides* was similar, and the infestation rate did not exceed 35%. The most abundant was *F. proliferatum*, which was identified in grains in greater quantities during 2020 when conditions were unfavorable. Among the minor causal agents of Fusarium wilt, the most abundant was *F. equiseti*, followed by *F. sporotrichioides*. However, in 2022, the high rate of infestation of corn kernels with *F. incarnatum*

was observed, the quantities of which reached the levels of *F. equiseti*. The amount of *A. parasiticus* was higher compared to *A. flavus*, although this species is more characteristic of the rhizosphere. In 2021, a decrease for *A. clavatus* was observed compared to 2020, and the average values of *A. clavatus* and *A. ochraceus* were almost identical. Environmental factors did not significantly affect the accumulation of *A. flavus* and *A. parasiticus* in grains. In maize leaves, the most common species of *Penicillium* were *P. chrysogenum* and *P. expansum*, followed by *P. citrinum* and *P. griseofulvum*. The quantities of *P. chrysogenum* were uniform during the years 2020-2022 and did not deviate significantly from the average value for the observation period. In 2021, when the decrease in the frequency and amount of *Penicillium* fungi in corn leaves was observed, this species showed the highest amounts.

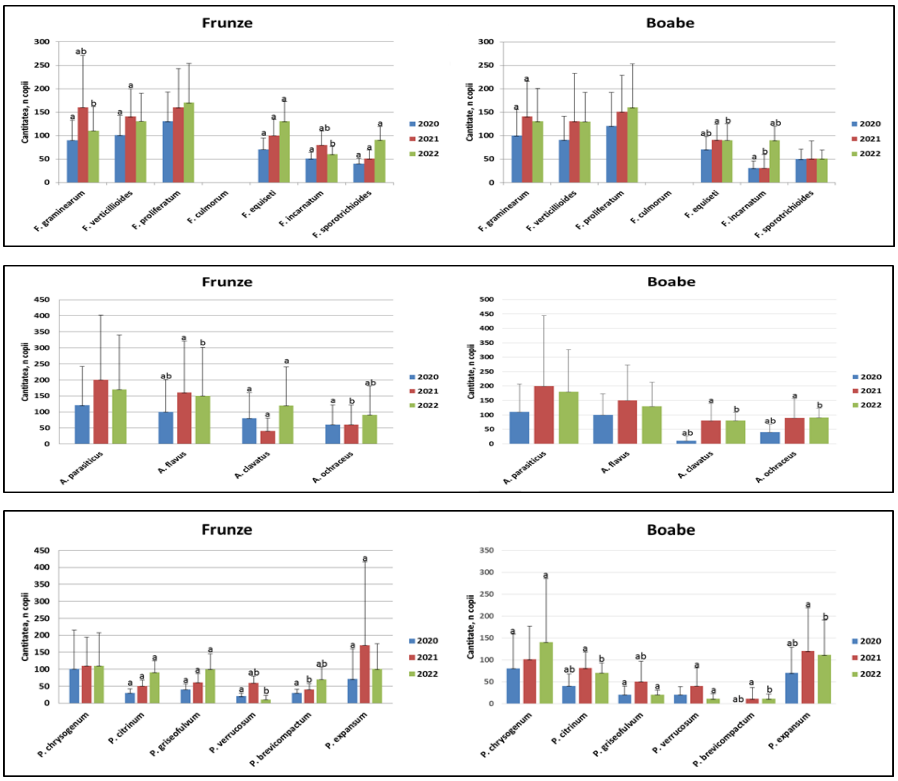

Fig.4.2. Average amounts of fungi in maize leaves and kernels (number of copies per 100 mg of plant material). Letters indicate values that differed significantly at p<.05

Only for *F. graminearum*, *F. sporotrichioides*, *P. expansum* and *A. flavus* was observed a strong positive correlation established between the amount of fungi in soil, grain and leaves, indicating that soil is the primary source of maize infection with these species. For other species the correlation between the amount of fungi in soil and plant organs was uneven. The correlation between concentrations of *Fusarium*, *Aspergillus* and *Penicillium* fungi in soil and their accumulation in corn shows that during the monitoring period several external factors acted on the propagation of the respective fungi in the host plant: the susceptibility of the host plant, climatic

factors , competition between species in the microbiome of the plant, the phytosanitary status of the seed material. In the case of *F. graminearum*, *A. clavatus*, *P. expansum*, *P. chrysogenum*, a strong correlation was observed between the concentration of these fungi in the soil, systemic infections of maize plants and accumulation in grains. All species are active producers of dangerous mycotoxins: fumonisins, aflatoxins, ochratoxin A, patulin. Thus, the rhizosphere microbiome plays an essential role in the propagation of these species in plants. In this case, improving the phytosanitary status of the soil can lead to a decrease in the rate of corn infestation with the given fungi.

Identification of toxigenic fungi in the seed material stored in the Gene Bank of the IGPPP. Eleven fungal genome sequences associated with the production of aflatoxins, fumonisins, zearalenone, T-2 and DON in different fungi of the genera *Aspergillus* and *Fusarium* were identified and quantified in maize kernels after storage in the IGPPP Gene Bank. *Aspergillus* and *Penicillium* species capable of producing ochratoxin A and patulin were not identified in the seed material. The analysis of the frequency of infection showed that 30.4% of the stored maize grains were infected with at least one toxigenic strain of pathogenic fungi, while in only 4% of the samples none of the fungal genes associated with the production of mycotoxins. In addition, one or two strains of toxic fungi were identified in about 4% of the analyzed samples, in 8% the mixed infection of 3, 5 and 6 strains was observed, and in about 21% of the samples the mixed infection of 4, 7 and 8 toxigenic fungal strains was observed. In general, the predominance of toxigenic strains of *Fusarium spp*. and *Aspergillus spp*. was observed in corn grains, and their co-occurrence was identified in 70.8% of analyzed grains. Toxic fungi have also been detected in both pollen and silk. The frequency of pathogens was higher in pollen samples compared to silk – 35.7% and 13.1%, respectively. The presence of fumonisin and aflatoxin producers in silk represents a significant risk for grain infection and subsequent mycotoxin contamination, as propagation through stigmas gives the pathogen the ability to bypass the natural barrier of maize cobs. In addition, pollen itself can act as a natural vector for the spread of fungi in dry weather cornfields. Average Cq values for most fungal sequences associated with mycotoxin production ranged from 31 to 36, indicating medium to low concentrations of target DNA (Fig.4.3). However, the dispersion of Cq values differed significantly for each identified gene. About 33% of the samples showed a high content (Cq<29) of *FUM6* specific for *F. verticillioides* and *FUM1* common for several *Fusarium* toxigenici species, while 20.8% of the samples showed a high content of *FUM6* specific for toxigenic *F. proliferatum* and *aflQ* specific for aflatoxin-producing *A. flavus* strains. Only a few samples showed accumulation of *TRI11* and *TRI8* sequences in low concentrations. The relative amount of *F. equiseti* able to produce zearalenone was insignificant.

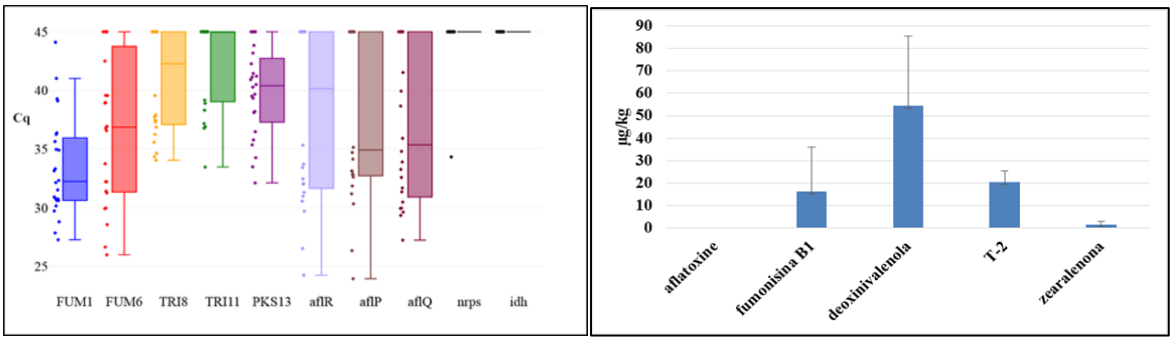

Fig. 4.3. Boxplot of mean Cq values of genes associated with mycotoxin production in fungi (left). Mean concentrations of aleaflatoxins, fumonisins, deoxynivalenol, T-2 toxin and zearalenone in maize kernels after six months of storage, obtained by ELISA (right)

A very strong correlation was established between the amount of mycotoxins and the amount of genes associated with mycotoxin biosynthetic pathways for aflatoxins (r=0.98) and zearalenone (r=0.92). For fumonisins a moderate positive correlation was established (r=0.6), and for DON and T-2 the given parameter was negative (r=-0.76 and r=-0.95 respectively). The different values of the Pearson coefficient can be explained by the different role of mycotoxins in fungal biology. Fumonisins are associated with the pathogenesis and facilitate the infection of plants by some *Fusarium* species, while aflatoxins act as antioxidants and increase the survival capacity of *Aspergillus* fungi under stress conditions, and zearalenone inhibits the propagation of fungi from solid media. The latter may have an important role for the competition of fungi under conditions of active propagation. Thus, with the amount of toxigenic fungi correlates the amount of those mycotoxins, which play an important role in the propagation and resistance of fungi to abiotic and biotic factors. The mycotoxins DON and T-2 also play an important role in fungal ecology; T-2 confers survival advantages and DON facilitates propagation of the fungus beyond the initial infection zone. The trichothecene biosynthetic cluster directs the synthesis of these mycotoxins. It can be assumed that toxigenic *Fusarium* species identified in grains are capable of synthesizing several mycotoxins from this family, and their population is heterogeneous and there is an interaction between the species, which results in different amounts of mycotoxins and the different correlation between the number of genes and concentration of secondary metabolites.

Thus, the grains obtained from maize plants grown on the experimental plots did not accumulate significant levels of mycotoxins. However, all tested samples accumulated medium and high amounts of *afl* and *FUM* gene clusters, indicating the presence of aflatoxin- and fumonisin-producing fungi in the grain. Co-occurrence of *Aspergillus spp*. and *Fusarium spp*. was the most widespread and observed in more than 70% of the grains analyzed. This means that the corn kernels before storage are infected with fungi that are capable of producing dangerous mycotoxins even in drought conditions that are not favorable for fungal growth. However, if strict

storage conditions are not observed, these fungi are capable of multiplying rapidly and contaminating corn kernels with fumonisins and aflatoxins. Therefore, the corn kernels obtained under the conditions of the experimental fields present a certain toxic risk. Preliminary screening tests performed in the current work suggest that a thorough monitoring of the risks associated with mycotoxin exposure using more accurate and sensitive detection methods in maize fields is needed.

The impact of maize genotype on the propagation of pathogenic fungi in plant organs. Moisture and free water are critical for fungal growth. Thus, plant morphophysiological parameters associated with rapid water release by cobs can serve as indices of constitutive resistance to fungi. In this sense, several characters, associated with water release, were selected to describe the maize genotypes of interest: flowering-maturation period, cob coverage with husks, cob to husk length ratio, cob diameter, number of rows of grains per cob (Tab.4.1). The flowering-maturity period shows how quickly maize plants go from silk emergence to full grain maturity, and the shorter this period, the lower the risk of infection. Full, uniform and thick covering of the inflorescence with husks saves underdeveloped grains from fungal infection, and partial covering makes the ear susceptible to fungus. However, too dense and thick cover prevents the natural drying of the cobs during ripening, and excess moisture creates a favorable environment for fungal development. In this sense, the mentioned parameter is closely related to the ratio between the length of cob and husks - when husks are longer than the cobs, this fact facilitates a greater retention of moisture under the cover leaves, which induces the active propagation of fungi. On the other hand, shorter panicles make the ears defenseless against phytopathogens. In general, the optimum ratio is 1:1, when the husks and cobs are of equal length. The smaller the diameter of the cob, the faster the corn kernels and cobs lose their moisture. A thinner cob releases water faster compared to a thicker one, therefore, the lower the values of the corresponding parameter, the lower the risk of moisture retention by the cob and the occurrence of fungal rot.

Table 4.1. The values of some morpho-physiological indices of maize genotypes

	Flowering-ripening period, days*	Cob coverage with husks, points**	The ratio of cob to husk lengths, points***	Ear diameter, mm	Number of grain rows per cob, n	Cob diameter, mm
MAN2308	**53**	**4**	**2**	**47**	**14**	**36**
MAN2452	**56**	**5**	**2**	**50**	**16**	**37**
MAN2414	**56**	**5**	**2**	**36**	**14**	**25**
MAN2413	**56**	**5**	**2**	**39**	**14**	**29**
MAN2451	**56**	**5**	**1**	**37**	**14**	**26**
MAN2424	**53**	**5**	**2**	**39**	**14**	**26**
MAN2425	**53**	**5**	**2**	**36**	**12**	**25**
MAN2459	**53**	**5**	**2**	**34**	**16**	**25**
MAN2448	**65**	**7**	**3**	**40**	**16**	**30**
MAN2281	**49**	**5**	**2**	**35**	**14**	**26**
MAN2461	**53**	**4**	**1**	**33**	**12**	**24**
MAN2526	**56**	**5**	**2**	**46**	**16**	**33**
CP148	**45**	**5**	**2**	**33**	**16**	**23**
CP137	**43**	**4**	**2**	**35**	**14**	**24**
MK01	**46**	**5**	**2**	**40**	**16**	**28**
Ku123	**52**	**5**	**2**	**38**	**12**	**28**
B73	**69**	**5**	**2**	**43**	**14**	**32**
MAN2488	**48**	**4**	**2**	**34**	**12**	**23**
MAN2493	**56**	**5**	**2**	**38**	**14**	**27**
MAN2491	**53**	**5**	**2**	**31**	**14**	**22**
MAN2483	**50**	**5**	**2**	**37**	**12**	**28**
MAN2470	**53**	**5**	**2**	**30**	**14**	**20**
MAN2466	**53**	**5**	**1**	**39**	**12**	**26**
MAN2463	**53**	**4**	**3**	**30**	**12**	**19**

** the period from the end of ear flowering to the physiological maturity of the kernels*

*** 3 – unsatisfactory coverage, 5 – intermediary coverage, 7 – good coverage [20]*

**** 1 – the length of the ear exceeds the length of the husks, 2 – the lengths of the ear and the husks are equal, 3 – the ear is shorter than the panicles*

The maize genotypes studied were characterized by an average flowering-maturity period of 53 days, intermediate ear coverage with a ratio of approximately 1:1 between cob and husk lengths, average ear diameter of 37.5 mm and an average of 13.9 kernel rows per cob. Maize B73, selected as a reference genotype for its susceptibility to fungal pathogens, showed the longest flowering-ripening period – 69 days. This genotype had intermediate values of cob thickness and satisfactory panicle coverage, while panicle and cob length were equal. The inbred line MAN2448 also showed a relatively long period between the end of cob flowering and grain ripening and showed better inflorescence coverage with panicles compared to the control line and had a thinner cob. However, the husks were longer than the cobs. Other lines were characterized by a shorter flowering-maturation period. The thickest cobs were observed for lines MAN2452, MAN2308 and MAN526. For other genotypes, this parameter was ≤40 mm. The thinnest cobs were recorded for genotypes MAN2470 and MAN2463. Short husks were observed for MAN2466, MAN2461 and MAN2451 maize. Another genotype with longer husks compared to cobs was line MAN2448.

Molecular analysis demonstrated that in mature corn kernels the species of major pathogenic fungi and storage fungi of the genera *Fusarium, Aspergillus* and *Penicillium* were present, associated with fungal diseases and grain damage (Fig. 4.4). The genotypes showed different values of the frequency of grains infected with fungi, and according to this parameter

three groups were highlighted: group I with infection rate <10% (resistant), group II –10-25% (tolerant), group III – infection rate greater than 25% (susceptibles).

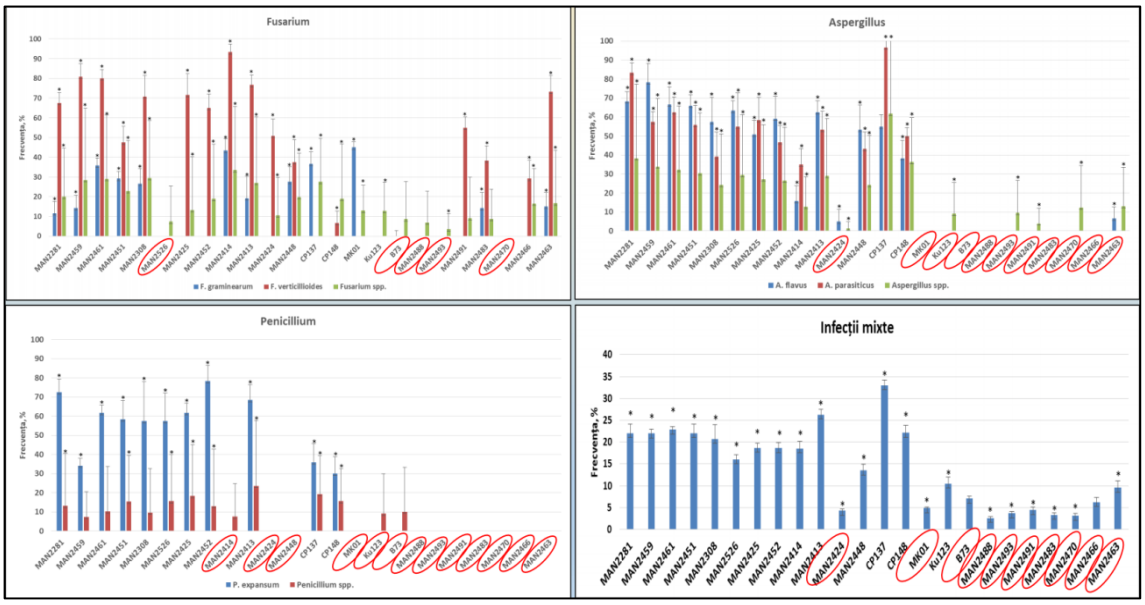

Fig.4.4. Frequency of corn kernels infected with fungal pathogens. The asterisk indicates the significant difference from the respective values of the genotype-standard at p<.05

Not all the fungal species identified in the soil and plants on the experimental fields were identified in the samples of the 24 genotypes taken in the study. Five genotypes were classified as resistant to fusariosis caused by several species of *Fusarium*, nine were shown to be resistant in *F. verticillioides*, 12 – in *F. graminearum*. Out of 24 genotypes, 11 showed low accumulation of *A. flavus* and *A. parasiticus*. Most genotypes showed low accumulation of *Penicillium* fungi. According to the mixed infection rate, 10 genotypes showed resistance to fungal accumulation. The species *F. culmorum*, *A. clavatus*, *P. chrysogenum*, *P. griseofulvum* and *P. verrucosum* were absent in the samples of the analyzed genotypes. It is observed that the standard genotype was classified as resistant, while most of the inbred lines showed tolerance to fungal pathogens.

The semi-quantitative evaluation of the accumulation of fungi in the grains was carried out based on the Cq values, and the genotypes were divided into groups according to the degree of susceptibility, as follows: Cq≤29 – high susceptibility, 29<Cq<37 – medium susceptibility, Cq≥ 37 – low susceptibility (Tab.4.2). *F. incarnatum*, *F. sporotrichioides*, *A. ochraceus* and *P. brevicompactum* were identified in very small quantities. The infection rate of the host plant with the other pathogens was genotype-dependent. No significant amounts of *F. graminearum* were identified in the grains of the standard genotype, as in CP148, MAN2424, MAN2452, MAN2425 and MAN2526. Although other genotypes showed statistically higher rates of infection with the given pathogen compared to the standard, but the DNA concentrations in the grains were low or medium, thus, it is not possible to conclude about the susceptibility of these genotypes based only

on the data presented. The grains of the standard genotype also showed high concentrations of a causative agent of FER – *F. verticillioides*. The genotypes MK01, Ku123, CP137 and MAN2526 were found to be weakly susceptible to the mentioned pathogen. Very small amounts of DNA of the given fungus were recorded in the grains of genotype CP148. A large amount of pathogenic DNA was found in samples from the inbred lines MAN2281, MAN2459, MAN2461, MAN2308, MAN2425, MAN2414 and MAN2413. The degree of susceptibility of the studied genotypes to *F. proliferatum*, another causative agent of FER rot, was lower compared to the previously named species. Most samples contained moderate or low levels of this pathogen. High DNA concentrations were found only in MAN2459 grain samples, this genotype also showed high susceptibility to *F. verticillioides* infection. On the contrary, other genotypes in which high concentrations of *F. verticillioides* were found (MAN2481, MAN2461, MAN2308, MAN2425, MAN2414 and MAN2413) were less susceptible to the fungus *F. proliferatum*. The inbred lines were found to be virtually immune to *F. equiseti* infection and very low concentrations of fungal DNA were found in the grains of CP137, CP148, MK01, Ku123 and the standard line B73. *F. incarnatum* DNA was found in significant amounts in the grains of only one genotype, cultivar CP148.

Table 4.2. Relative amount of major causative agents of Fusarium wilt and some fungi associated with damage to maize kernels during storage (selective data)

Fungi / Genotype	FG	FV	FP	AF	AP	PCt	PE
MAN2281	37.31	28.88	35.66	28.81	26.34	45.00	28.92
MAN2459	39.56	26.38	28.21	28.35	31.86	39.15	36.02
MAN2461	35.58	27.51	31.34	29.88	30.34	45.00	31.39
MAN2451	37.46	33.75	31.36	30.90	32.62	36.45	31.07
MAN2308	37.44	28.02	32.86	31.94	34.15	45.00	30.21
MAN2526	45.00	45.00	32.35	30.65	31.19	34.66	32.01
MAN2425	45.00	27.63	40.90	32.87	32.83	34.81	31.11
MAN2452	45.00	30.42	33.17	32.16	33.14	45.00	28.68
MAN2414	34.05	25.47	36.41	38.95	35.15	34.63	45.00
MAN2413	37.75	28.01	30.99	30.24	32.83	29.46	29.85
MAN2424	45.00	33.71	39.39	40.78	45.00	45.00	45.00
MAN2448	37.91	35.06	31.19	32.14	34.70	45.00	45.00
CP137	36.87	45.00	31.55	31.28	23.95	34.51	35.01
CP148	45.00	40.02	45.00	34.38	33.09	37.56	36.31
MK01	34.36	45.00	45.00	42.69	45.00	45.00	45.00
Ku123	45.00	45.00	38.37	41.62	45.00	31.68	45.00
B73	45.00	45.00	37.98	45.00	45.00	30.54	45.00
MAN2488	45.00	45.00	34.14	45.00	45.00	45.00	45.00
MAN2493	45.00	45.00	45.00	45.00	45.00	45.00	45.00
MAN2491	45.00	32.83	45.00	45.00	45.00	45.00	45.00
MAN2483	38.31	34.16	45.00	45.00	45.00	45.00	45.00
MAN2470	45.00	45.00	45.00	45.00	45.00	45.00	45.00
MAN2466	45.00	45.00	45.00	45.00	45.00	45.00	45.00
MAN2463	38.19	28.57	45.00	39.73	45.00	45.00	39.30

FG – F. graminearum; FV – F. verticillioides; FP – F. proliferatum; AF – A. flavus; AP – A. parasiticus;PCt – P. citrinum;PE – P. expansum

Cq≤29 values indicate relatively high amount of DNA (red); 30<Cq<37 – average relative quantity (yellow); Cq≥37 – low relative amount or absence of the pathogen (green)

For fungal species identified with multiple primer pairs, average Cq values are given

Another pathogen, *F. sporotrichioides*, was found only in CP137 and Ku123 grains, but in small amounts, this fungus was not found in grain samples of other genotypes and the standard line B73. Two important species of *Aspergillus* fungi associated with grain spoilage and aflatoxin contamination were mainly found in medium concentrations. The genotypes MAN2459, MAN2461 and CP137 were the most susceptible to the fungus *A. flavus*. The grains of the inbred line MAN2281 were found to be sensitive to both fungal species. In addition, the highest concentrations of *A. ochraceus* DNA were found in the grains of cultivar CP137, while the standard genotype was found to be weakly susceptible to infection by fungi of the genus *Aspergillus*. Compared to *Fusarium* and *Aspergillus* fungi, pathogens of the genus *Penicillium* were present in the grains of the studied genotypes in much smaller quantities. Genotype MAN2413 was the most sensitive to the fungi *P. citrinum* and *P. expansum*. The highest amount of *P. expansum* was found in MAN2281 and MAN2452 grains. Thus, the maize genotypes studied proved to be the most susceptible to *F. vericillioides* infection, which was found in high concentrations in seven lines. Line MAN2281 proved to be the most sensitive to infection with pathogenic fungi - four types of microorganisms associated with systemic infections, spoilage of grains during storage and contamination with mycotoxins were found in high concentrations in samples of this genotype. Line MAN2459 also showed high sensitivity, in grain samples from which two main causative agents of cob fusarium and the main producer of aflatoxins were found. The standard line proved to be weakly sensitive to the pathogens studied under the conditions of the Republic of Moldova.

The infection rate and the spectrum of toxigenic pathogens were different in the analysed maize genotypes. About 5 different fungal sequences associated with the synthesis of different mycotoxins were simultaneously identified in the analysed grains. The highest sequence diversity of toxigenic clusters was identified in cultivars CP137, CP148 and six inbred lines MAN2451, MAN2526, MAN2425, MAN2452, MAN2414 and MAN2413 (six sequences). But the average Cq values ranged between 35-40, which corresponds to low concentrations of the DNA of interest in the analyzed samples. The least infected were the lines MK01, Ku123 and the standard line – three sequences associated with the synthesis of mycotoxins were identified in the grains of these genotypes with average values of Cq 42-43. That is, DNA of practical interest was absent from the analyzed samples.

The grains of MAN2459, MAN2452 and MAN2414 were the most infected with toxigenic *Fusarium* species capable of producing fumonisins. CP148, Ku123, B73 and MK01 grains were the least prone to infection with fumonisin-producing species, while MAN2481 and MAN2459 showed the highest amount of *F. proliferatum*-specific *FUM1* sequence. The highest amounts of

FUM6 gene sequences specific for toxigenic *F. verticillioides* and *F. proliferatum* and *FUM1* sequence specific for several fumonisin-producing *Fusarium* species were simultaneously identified in MAN2459 grains. No toxigenic *F. equiseti* strains capable of producing zearalenone were identified in samples of this genotype. *PKS13* gene sequence showed the highest Cq values in MAN2413, MAN2451 and MAN2414 grains. The lowest amount of this sequence was identified in maize kernels of Ku123 and B73.

In the grains of line B73 the sequences of the *afl* gene cluster were absent, while in the grains of Ku123 and MK01 only the *A. flavus* specific *aflQ* sequence was identified. MAN2281 and CP137 were the most susceptible to infestation with toxigenic *Aspergillus* fungi - the highest Cq values of *aflP*, *aflQ* and *aflR* genes were calculated for the grains of these lines. CP137 and MAN2281 were more susceptible to infection by aflatoxin-producing *Aspergillus* compared to fumonisin-producing *Fusarium* species. The total amount of trichothecene biosynthetic cluster gene sequences was significantly lower compared to fungal genome sequences associated with fumonisin and aflatoxin production. The highest Cq values of *TRI* sequences were calculated for MAN2461, MAN2414, CP137 and MK01. The most abundant was the *TRI8* gene sequence, while the *TRI11* sequence specific for toxigenic *F. sporotrichioides* was only identified in MAN2414. No sequences associated with T-2 and DON production were identified in MAN2452, MAN2526, MAN2425, MAN2424, Ku123 and CP148. Therefore, all grain samples stored in the IGPPP Gene Bank were infested with different toxigenic fungi capable of producing dangerous mycotoxins under certain conditions. Fumonisin - and aflatoxin-producing species of the genera *Fusarium* and *Aspergillus* were predominant in maize grain samples. The impact of maize genotype on the amounts of *FUM* and *afl* sequences in maize kernels was observed.

The genetic polymorphism analysis of the studied genotypes was carried out using primers for genomic markers from the class of mobile elements (Fig.4.5).

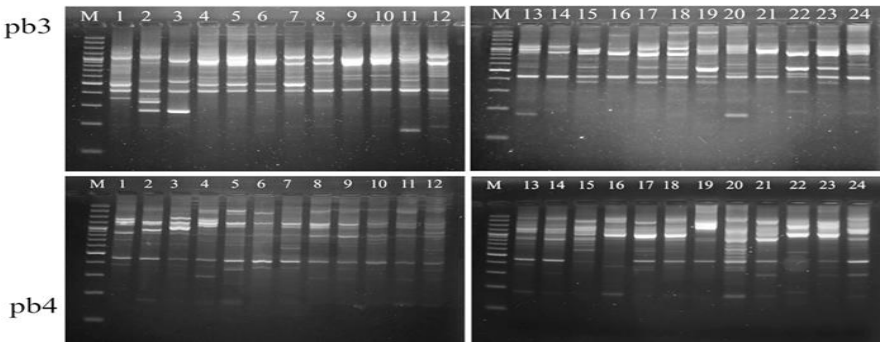

Fig.4.5. Example of genetic profiles generated from amplification of maize DNA with primers pb3 and pb4: 1 – CP137, 2 – CP148, 3 – B73, 4 – Ku123, 5 – MK01, 6 – MAN2526, 7 – MAN2448, 8 – MAN2459, 9 – MAN2413, 10 – MAN2466, 11 – MAN2451, 12 – MAN2463, 13 – MAN2452, 14 – MAN2461, 15 – MAN2488, 16 – MAN2308, 17 – MAN2491, 18 – MAN2281, 19 – MAN2424, 20 – MAN2425, 21 – MAN2483, 22 – MAN2493, 23 – MAN2414, 24 – MAN2470, M – molecular marker GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific

These markers are dominant, universal and distinguished by high reproducibility. From the primers used for the analysis of the genetic variation of the maize genotypes, positive amplification reactions were recorded with only six oligonucleotide sequences. Only clearly visible bands were considered for analysis. The specific bands, generated by primers based on mobile elements, are of interest as a source of genomic information for the development of marker systems for maize traits of interest. The primers generated distinct and reproducible patterns (2-3 repeats) with an average PIC value of 0.45 – near maximum for dominant markers (Tab. 4.3). The average rate of polymorphism was also high and the respective value constituted 92.67%. Six primers in total generated 122 bands, of which 80 were polymorphic and 18 – unique. The number of bands generated per genotype varied between 3.83 and 7.73 with an average rate of 5.93 per genotype. The minimum values of the respective parameter were recorded for the MAN2424 line (3.67 amplicons), and the maximum values for the MAN2526 line (seven distinct bands). The best performance was shown by primer D2 developed based on the Activator sequence, for which the highest levels of polymorphic and unique bands were recorded – 32 and 6 bands respectively. For the given primer, the lowest PIC values were calculated compared to sequences from the iPBS class, but the high rate of polymorphism and the ability to generate unique patterns resulted in a high value of the marker index – 8.21. In addition, high performance was shown by primer pb5. Although this sequence did not generate specific amplicons, but the high rate of polymorphism allowed obtaining individual amplification patterns, which resulted in the high efficiency of the sequence used. The lowest performance was shown by primer pb6. The lowest polymorphism and marker index MI values were calculated for that sequence due to a limited number of bands, one of which was monomorphic. Primer pb20 was more effective for detecting genetic diversity compared to pb6 with near-maximum PIC value for dominant markers, but the limited number of

bands did not make it possible to generate more specific amplification patterns as observed for D2. The last two primers pb3 and pb4 generated an equal number of amplicons and showed high polymorphism values. Primer pb4 showed the highest value of the MI marker index.

Table 4.3. The values of the essential descriptors for the analysis of the effectiveness of the primers, calculated following the experimental amplification with DNA of different maize

Primer / Index	Pb3	Pb4	Pb5	Pb6	Pb20	D2	Mean
Lenght, pb	3072-165	2240-175	1971-178	1256-287	2908-277	3100-205	2424-214
N	22	22	17	10	19	32	20.33
NM	1	0	0	1	0	0	0.33
NP	21	22	17	9	19	32	20
NS	5	3	0	0	3	6	2.83
Z	6.04	6.75	6.41	3.83	7.73	4.8	5.93
PP, %	94.45	100	100	90	100	100	97.41
PIC	0.39	0.43	0.47	0.47	0.44	0.26	0.41
R	12.08	13.5	12.83	7.67	12.25	9.67	11.33
E	20.04	22	17	8.1	19	32	17.68
MI	7.98	9.36	7.98	3.83	8.31	8.21	7.61
D	0.32	0.4	0.45	0.36	0.37	0.26	0.36
n_a	1.95	2	2	1.9	2	2	1.98
n_e	1.29	1.37	1.48	1.32	1.36	1.18	1.33
h	0.19	0.25	0.29	0.22	0.23	0.14	0.22

N – total number of bands; NM – monomorphic bands; NP – polymorphic bands; NS – specific bands; Z – average number of bands per genotype; PP – polymorphism rate; PIC – polymorphic information content; R – the resolving power of the primer; D – the discrimination power of the primer; E – effective multiplexing rate; MI – marker index; na – the number of observed alleles; ne - the number of effective alleles; h – Nei genetic diversity

Thus, the most specific sequences were detected in the MAN2425 genotype (four specific loci). For the corn genotypes studied, values of observed alleles na=1.98±0.22, effective alleles ne=1.33±0.28 were found. The Nei genetic variation index was h=0.22±0.14, and for the informative index quite low values of D=0.36±0.19 were found, which indicates high variability and the efficiency of the primers for the analysis of intergenotypic variation.

The generated amplicons were used for the grouping of maize genotypes based on the Jaccard coefficient (the highest cophenetic coefficient – 0.76) by the UPGMA method (Fig.4.6). The smallest genetic distances are observed between Ku123 and MAN2459. The dendrogram based on the genetic variation grouped the genotypes in cluster II containing the varieties CP137 and CP148, and the inbred lines were distributed in cluster I, which is divided into several heterogeneous subclusters. These cultivars generated identical genetic profiles with the pb6 primer, and the similarity rates of the patterns generated by the other primers varied between 72-90%. The standard-genotype B73 was assigned to a subcluster together with MK01, Ku123 and the varieties CP137 and CP148. These genotypes showed reduced rates of fungal DNA accumulation in kernels. Among the inbred lines, similarity was observed between the genetic profiles of MAN2414-MAN2424, MAN2461-MAN2459, MAN2281-MAN2451, MAN2448-

Ku123, which may suggest a certain degree of kinship based on the common progenitor. The control line B73 shows some genetic similarity to lines MAN2414 and MAN2424.

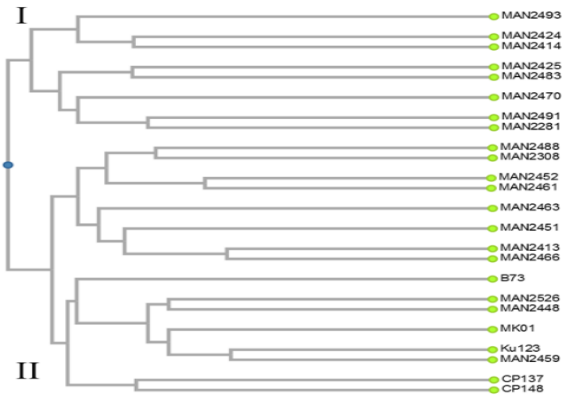

Fig.4.6. UPGMA clustering of maize genotypes based on genetic polymorphism

Certain polymorphic amplicons generated with primers derived from mobile element sequences were detected at high frequencies (more than 50%) in the group of resistant genotypes compared to tolerant and susceptible ones. In this sense, the following sequences are of interest as a potential source of resistance:

pb3 – 813 pb (56%), 596 pb (67%), 522 pb (67%), 487 pb (56%);

pb4 – 860 pb (63%), 445 pb (87%);

pb20 – 948 pb (87%), 768 pb (63%), 401 pb (63%);

pb5 – 980 pb (87%), 578 pb (75%), 402 pb (63%);

pb6 – 880 pb (63%), 382 pb (75%).

The distance matrix values, calculated based on the Euclidean distance coefficient, were not used for UPGMA clustering, which revealed the level of similarity between genotypes and the relationship between genetic variation and characters of interest (Fig.4.7). The grouping of genotypes was carried out according to genetic variation characters, morphophysiological indices associated with resistance to fungal infections, infection rates with species of fungal pathogens from the genera *Fusarium*, *Aspergillus*, *Penicillium*. Likewise, clustering was performed according to multiple characters: combination of genetic variation and morphophysiological indices, genetic variation and the rate of infection of corn grains with fungal pathogens, genetic variation overlapped with morphophysiological indices and the rate of infection of grains with fungal pathogens. In each case, two uneven clusters were observed and the distribution of genotypes in subclusters depending on the chosen parameter.

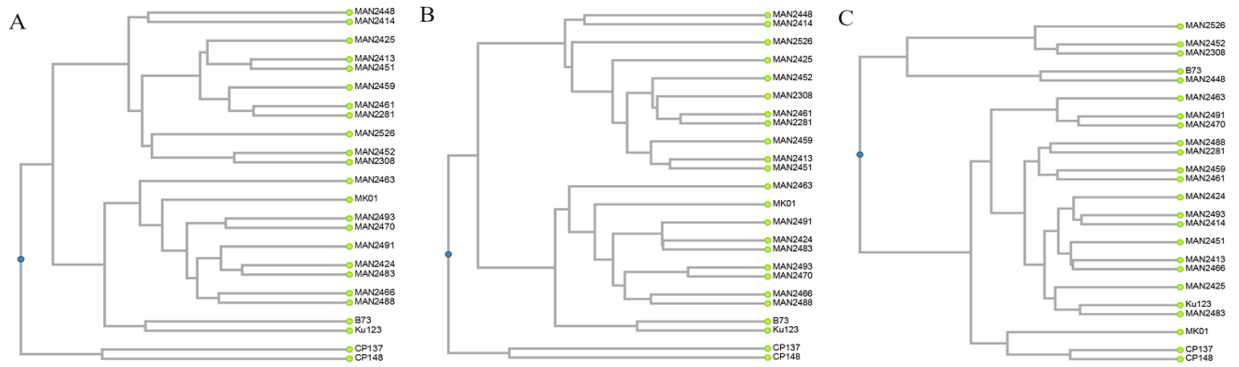

Fig. 4.7. UPGMA dendrograms of maize genotypes under genetic variation overlaid with morphophysiological indices and grain infection rate with fungal pathogens (A), genetic variation and maize grain infection rate with fungal pathogens (B), combination of genetic variation and indices morphophysiological (C)

The uniform distances between genotypes were observed for clustering based on morphological characters and genetic polymorphism, while for other grouping criteria the degree of similarity between maize plants was more heterogeneous. The formation of similar subclusters is observed based on grouping by several parameters. CP137 and CP148 genotypes were assigned to a cluster by all three clustering parameters. Genotypes MAN2448 and MAN2414 also cluster together by this parameter, as does Ku123-B73. Grouping by three listed parameters and the combination of genetic polymorphism-pathogen accumulation rate in grains form a practically identical subcluster, which includes genotypes MK01, MAN2463, MAN2488, MAN2466, MAN2483, MAN2424, MAN2491, MAN2470, MAN2493. This cluster was not formed in the case of grouping according to the parameter of genetic variation, morphophysiological indices or the association between genetic and morphophysiological characters. It can be deduced that the genotype has a significant impact on the rate of infection with fungal pathogens in the analyzed corn.

Conclusions and recommendations

In the conducted study, the following general conclusions were made:

1. The analysis protocol based on molecular methods was effective for monitoring the causal agents of corn fungal diseases in soil and plant material. Primers developed *de novo* based on fungal genome sequences allowed the precise identification of microorganisms that induce maize diseases during the growing season and yield damage during storage. Using the mentioned primer set, the following species were identified: *F. graminearum, F. verticillioides, F. proliferatum, F. culmorum, F. equiseti, F. incarnatum, F. sporotrichioides, A. parasiticus, A. flavus, A. ochraceus, A. clavatus, P. chrysogenum, P. expansum, P. citrinum, P. griseofulvum, P. verrucosum, P. brevicompactum*. Most of the designed primers have been successfully used for

both conventional and real-time PCR analysis. With the help of primers developed based on the sequences of the genome of fungi associated with the synthesis of the main classes of mycotoxins, the microorganisms capable of producing aflatoxins, fumonisins, DON, ZEN, T-2 toxin were discriminated. The analysis procedures based on the conventional PCR method with the application of the Poisson distribution and consistent dilutions allowed the quantification of fungi and the highlighting of the degree of susceptibility of corn plants to fungal infections. Thus, these procedures can be used as an alternative to the real-time PCR method.

2. Out of 24 analyzed maize genotypes, 12 showed resistance to *F. graminearum*: – MAN2526, MAN2425, MAN2452, MAN2424, CP148, Ku123, B73, MAN2488, MAN2493, MAN2491, MAN2470, MAN2466. The genotypes MAN2526, CP137, CP148, MK01, Ku123, B73, MAN2488, MAN2493, and MAN2470 showed resistance to *F. verticillioides* infection. The following genotypes were shown to be resistant to mixed infections caused by several major and minor causative agents of fusarium: MAN2526, B73, MAN2488, MAN2493, MAN2470. Eleven maize genotypes showed resistance to *Aspergillus spp*.: MAN2424, MK01, Ku123, B73, MAN2488, MAN2493, MAN2491, MAN2483, MAN2470, MAN2466, MAN2463. More than half of the analyzed genotypes showed low levels of *Penicillium spp*. infection: MAN2414, MAN2424, MAN2448, MK01, Ku123, B73, MAN2488, MAN2493, MAN2491, MAN2483, MAN2470, MAN2466, and MAN2463. Ten genotypes were shown to be resistant to mixed infections by *Fusarium spp*., *Aspergillus spp*., *and Penicillium spp*.: MAN2424, MK01, B73, MAN2488, MAN2493, MAN2491, MAN2483, MAN2470, MAN2466, and MAN2463.

3. Molecular analysis revealed the unsatisfactory phytosanitary status of experimental maize fields and germplasm stored in the IGPPP Gene bank. The main causative agents of fusarium, aspergillosis and penicillosis in maize, as well as major fungal contaminants with aflatoxins, fumonisins and trichothecenes have been identified in soil, plant leaves and stored grain. Therefore, the soil and the seed material itself are the source of subsequent infection of maize plants during the growing season. There was an uneven impact of abiotic factors on fungal propagation. Mostly, *Aspergillus* and *Penicillium* species associated with grain spoilage and some minor causal agents of fusarium wilt in maize plants were more significantly affected by fluctuations in external environmental factors. For fungi that are part of the soil microbiota, no specific correlation has been established between their sensitivity to air temperature and humidity fluctuations and its ecological particularities.

4. For some classes of mycotoxins, a positive correlation was established between the Cq values of the fungal genes associated with mycotoxin biosynthesis and the actual concentration of the toxic metabolite in grain samples. Therefore, the Cq value can be used to monitor the

potential dynamics of mycotoxins in maize samples. Although critical Cq values corresponding to a certain concentration of mycotoxins in grain samples were not identified due to insufficient sample numbers, mostly Cq values around 33.8 already indicated the presence of mycotoxin in the sample.

Is recommended:

1. The use of the PCR protocol and tested primers for the monitoring of phytopathogens in special laboratories active in the fields of phytosanitary control, phytopathology and mycology.

2. The use of the molecular diagnostic procedures presented in the thesis for the practical training of students in the disciplines of genetics, molecular biology, modern biotechnologies.

3. The use of genotypes MK01, Ku123, MAN2424, MAN2470, B73 in programs to improve maize resistance to fungal diseases.

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Adnotare

Grăjdieru Cristina, ”Identificarea genetico-moleculară a genotipurilor de porumb (*Zea mays* L.) Rezistenți la unii patogeni fungici”, teză de doctorat în științe biologice, Chișinău, 2024

Structura tezei: introducere, 4 capitole, concluzii generale și recomandări, bibliografie din 236 de titluri, 116 de pagini de text de bază, 48 de figuri, 14 tabele, 2 anexe, declarație de proprie răspundere, CV. Rezultatele obținute sunt publicate în 20 lucrări științifice.

Cuvinte-cheie: PCR, porumb, *Fusarium*, *Aspergillus*, *Penicillium*, micotoxine.

Scopul: elaborarea de metode rapide și precise pentru evaluarea susceptibilității porumbului la agenții patogeni fungici în scopul selectării germoplasmei valoroase ca material de bază pentru programele de ameliorare a porumbului bazate pe rezistența la fungi din genurile *Fusarium, Aspergillus, Penicillium.*

Obiective: testarea unui set de primeri proiectați *de novo* pentru identificarea fungilor patogene și toxigenice asociate bolilor porumbului, evaluarea stării fitosanitare a câmpurilor experimentale de porumb și a germoplasmei de porumb stocate în Banca de gene al IGFPP, evaluarea eficacității markerilor în baza elementelor mobile pentru genotiparea porumbului.

Noutatea științifică: au fost evaluate soiuri și linii de porumb din Banca de gene a IGFPP și identificate genotipuri cu rezistență sporită la patogeni fungici.

Rezultatul obținut care contribuie la soluționarea unei probleme științifice importante constă în identificarea donatorilor de germoplasmă valoroasă pentru programele de ameliorare a rezistenței porumbului la fungi în baza metodei PCR.

Semnificația teoretică: a fost studiat impactul factorilor abiotici și al genotipului de porumb asupra dinamicii fungice în câmpurile experimentale de porumb și germoplasma stocată; a fost realizată analiza calitativă și cantitativă a principalelor producători de micotoxine din boabele de porumb; a fost stabilită corelația dintre acumularea de micotoxine și numărul de grupuri de gene asociate producției de micotoxine, a fost studiat polimorfismul ADN-ului de porumb.

Valoarea aplicativă: a fost propusă aplicarea markerilor moleculari și protocoalelor PCR pentru monitorizarea fungilor patogenici și toxigenici în culturi agricole și produse alimentare. A fost testată germoplasma de porumb din Banca de gene a IGFPP și au fost selectate genotipuri de perspectivă pentru programele de ameliorare.

Implementarea rezultatelor: rezultatele au fost prezentate la conferințe științifice naționale și internaționale, publicate în reviste științifice. Procedeele de diagnostic molecular expuse în teză au fost folosite pentru rezolvarea unor probleme aplicative: evaluarea stării fitosanitare a livezilor de mere din Republica Moldova și a eficacității mai multor condiții de depozitare a fructelor cu scopul reducerii deteriorării acestora cauzate de microorganisme; analiza complexă a acumulării micotoxinelor în produse alimentare pe parcursul depozitării; identificarea genotipurilor de tomate rezistente la fitoplasmă.

Annotation

Grajdieru Cristina, "Molecular identification of maize (*Zea mays* L.) genotypes resistant to fungal pathogens", PhD thesis in biological sciences, Chisinau, 2024.

Structure: introduction, 4 chapters, conclusions and recommendations, bibliography of 236 entries, 116 pages of body text, 48 figures, 14 tables, 2 appendices, personal responsibility declaration, CV. The results are published in 20 scientific papers.

Keywords: PCR, maize, *Fusarium, Aspergillus, Penicillium,* mycotoxins.

Scope: developing a PCR-based approach for assessing maize susceptibility to fungal pathogens of *Fusarium, Aspergillus* and *Penicillium* genera and selecting germplasm of interest for breeding maize resistance to fungal diseases.

Objectives: testing a set of primers designed *de novo* for identifying pathogenic and toxigenic fungi associated with maize diseases, assessing the phytosanitary status of experimental cornfields and maize germplasm stocked in Gene bank of, assessing the efficacy of markers based on mobile genetic elements for maize genotyping.

Scientific novelty: maize samples from the active collection of IGPPP were analyzed and genotypes with increased resistance to fungal pathogens were identified.

Thecontribution of the work to the solution of scientific problem is the identifying donors of valuable germplasm for maize breeding programs of resistance to fungi using PCR assays.

Theoretical significance: the impact of abiotic factors and maize genotype on fungal dynamics in cornfields and stored germplasm, qualitative and quantitative analysis of the main mycotoxin-producing fungi in maize grain, correlation between mycotoxin accumulation and number of gene clusters associated with mycotoxin production, maize DNA-polymorphism was studied.

Practical application: application of specific primers and PCR protocol for monitoring pathogenic and toxigenic fungi in agricultural and food products. Maize germoplasm from Gene bank of IGPPP was tested and perspective genotypes were selected for breeding programs of resistance.

Implementation of the results: the results were presented at national and international scientific conferences and published in scientific journals. The presented methods were applied to assess the phytosanitary status of apple orchards and determine the most effective storage parameters to reduce pathogen damage to apples; comprehensive assessment of mycotoxin accumulation in foods during storage; identification of tomato genotypes resistant to phytoplasma.

Аннотация

Грэждиеру Кристина, «Молекулярно-генетическая идентификация генотипов кукурузы (*Zea mays* L.) устойчивых к грибковым патогенам», диссертация на соискание степени доктора биологических наук, Кишинэу, 2024.

Структура: введение, 4 главы, выводы и рекомендации, список литературы из 236 ссылок, 116 страниц основного текста, 48 рисунков, 14 таблиц, 2 приложения, заявление о личной ответственности, CV. Результаты опубликованы в 20 научных работах.

Ключевые слова: ПЦР, кукуруза, *Fusarium, Aspergillus, Penicillium*, микотоксины

Цель: разработка метода ПЦР для оценки восприимчивости кукурузы к грибковым патогенам родов *Fusarium, Aspergillus* и *Penicillium* для выявления ценной зародышевой плазмы с целью селекции по признакам усточивости к грибковым болезням.

Задачи: тестирование набора праймеров, разработанных *de novo* для идентификации патогенных и токсигенных грибов, оценка фитосанитарного статуса экспериментальных полей кукурузы и зародышевой плазмы кукурузы, хранящейся в банке геновИГФЗР, оценка эффективности маркеров на основе мобильных элементов для генотипирования кукурузы.

Научная новизна: были проанализированы образцы кукурузы из коллекции ИГФЗР и выявлены генотипы с повышенной устойчивостью к грибковым патогенам.

Вклад работы в решение научных задач состоит в выявление ценной зародышевой плазмы для программ селекции кукурузы на устойчивость к грибам на основе метода ПЦР.

Теоретическое значение: были изучены влияние абиотических факторов и генотипа кукурузы на динамику грибов на кукурузных полях и в хранимой зародышевой плазме, качественный и количественный анализ основных грибов-продуцентов микотоксинов в зерне кукурузы, корреляция между накоплением микотоксинов и количеством кластеров генов, связанных с продукцией микотоксинов, ДНК-полиморфизм кукурузы.

Практическое применение: использование молекулярных маркеров и протокола ПЦР для мониторинга патогенных и токсигенных грибков в сельскохозяйственных и пищевых продуктах. Была протестирована зародышевая плазма кукурузы из коллекции Банка генов ИГФЗР и выбраны перспективные для селекции устойчивости генотипы.

Внедрение результатов: результаты представлены на национальных и международных научных конференциях, опубликованы в научных журналах. Изложенные методы были применены для оценки фитосанитарного состояния яблоневых садов и определения наиболее эффективных параметров хранения для снижения поражения яблок патогенами; комплексная оценки аккумуляции микотоксинов в пищевых продуктах во время хранения; выявления генотипов томатов, устойчивых к фитоплазме.

GRAJDIERU Cristina

MOLECULAR IDENTIFICATION OF MAIZE (*ZEA MAYS* L.) GENOTYPES RESISTANT TO SOME PATHOGENIC FUNGI

162.01. Plant genetics

Summary of PhD thesis in biology

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