Identification of Ulmus Pumila L. Pathogens with the Help of PCR

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***Ulmuspumila* L. (Ulmaceae) is one of the flora native species of Kazakhstan and forms the basis of dendroflora of all the populated areas. During the past few years there was registered the development of unknown diseases of Ulmuspumila. Molecural-genetic analysis stated that the pathogens of these diseases are microfungus of *Fusarium* – *F.solani* genus and *F.oxysporum* genus. Nowadays the investigations are aimed at finding out the microorganisms-antagonists of these phytopathogens.**

Key words: Phytopathogen, invasive blasts and diseases, Ulmus pumila, toxicity, micromycetes, antagonists, PCR.

Ulmus pumila L. (Ulmaceae) is one of the flora native species of Kazakhstan and nowadays forms the basis of dendroflora of all the populated areas. According to the results of investigations its share in wood communities of the region is 37.8%[1,2]. *U.Pumila* is a big deciduous fast-growing tree with a round or oval spreading thick crown. Ulmus Pumila is not very demanding to the soil conditions. Elms are often invaded by blasts and dangerous fungal diseases. Unfortunately the so-called Dutch elm disease often destroys a lot of elms. Despite this fact the development of the most dangerous diseases of this type of species has not been registered till past years in Kazakhstan. According to the literature sources *U. Pumila* is relatively resistant to the main disease of elms - Dutch elm disease, caaused by the fungus of Nectria (*Nectriacinnabarina* Phyl.) genus, which became the reason for mass mortality of the whole tree communities in some countries of Europe[3-6]. The unknown diseases of *U. Pumila* trees in dendroflora of Kazakhstan have been being registered in the past 10 years. It was stated that this process is connected with the invasion of the type of blasts-xylophages which are new for the country, their larvas are the main distributors of sporules of the phytopathogenic microbial flora. Invasive disease causes the putrefaction processes and cancer of *U. Pumila* stems which lead to the death[7,8]. Previous investigations did not define species composition of phytopathogens which cause diseases of trees. That's why the molecular-genetic identification of invasive diseases pathogens of the Ulmus Pumila was the task of our investigation.

Materials and methods of investigation

The material for the investigation was 2 strains of micromycetes, derived from the invaded tissues of *U.Pumila* stem (figure 1). Pure culture of the supposed phytopathogens was acquired by means of exhaustive subculture method on selective medium. The abstraction and registration of micromycetes were performed with the help of standard microbiological methods[9-12].

Identification of micro-organisms was

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performed by means of direct determination of nucleic acid sequence and their ITS regions following the comparison of their identity with nucleic acid sequences containing in the international samples database (GeneBank) as well as building of phylogenetic tree with nucleic acid sequences of the strains. DNA purification was performed in buffered solution with pH 8.0 which contained: 100 mMTris-HCl, 1,4 M NaCl, 20 mM EDTA, 2% CTAB and 100 mg/ml of proteinase. After centrifuging and removal of supernatant the investigated culture was grinded with the addition of liquid nitrogen. Then 100 mcl of the suspension of the culture was incubated during 18 hours with the addition of 500 mcl of the corresponding buffer. The cleaning of the suspension was performed with the addition of 750 mcl of chloroform/isoamyl alcohol (24/1), mixing and further centrifuging by 12 000 RMP during 10 minutes. The aqueous stage of the culture was then purified again with the help of phenol/chloroform/isoamyl alcohol (24/24/1). DNA residue was washed by 70%-ethanol with the further centrifuging and removal of the liquid stage. The acquired residue was dried in the air during 15 minutes. The samples of DNA were dissolved in 100 mcl of single TE buffer and stored under the temperature of -20°C. The DNA concentration was measured with the help of spectrophotometer NanoDrop having the wave's length of 260 nm[13].

The amplification of ITS region was performed in the reaction of PCR with the primers of ITS 5 5' – ggaagtaaaagtcgtaacaagg-3' and ITS4 5'-tcctccgcttattgatatgc-3' in the total volume of 30 mcl.

PCR amplification programme included the long-term denaturation under the temperature

Fig. 1. Type of UlmusPumila stems diseases

of 95°C during 4 minutes; 30 cycles under the temperature of 95°C during 25 seconds, 52°C - 30 seconds, 72°C - 40 seconds. The final elongation was performed under the temperature of 72°C during 7 minutes. PCR programme was performed with the use of DNA amplifier Engine Tetrad 2 CyclerPTC-0240 (Bio-Rad). Before the determination of nucleic acid sequence PCR products from unbounded primers were cleaned by means of fermentation using Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas). Sequencing reaction was performed with the use of BigDye® Terminator v3.1 CycleSequencingKit (Applide Biosystems) (in accordance with the manufacturer's instruction) with the further separation of segments of the automated genetic analyzer3730 xlDNAAnalyzer (Applide Biosystems).

Phylogenetic tree of the investigated strains of microfungi was built with the help of Mega 5.0 software using ClustalW algorithm for the alignment of the nucleic acid sequences, and using Neighbor-Joining NJ programme.

Investigation results. The study of the fungi's cultural characters in pure cultures showed that the 2 studied strains can be ranged in a class of imperfect. These strains with the well developed

myceliums have formed convex colonies with the diameter of 1.5-2.0 cm on the nutritious medium. On the 10th day of the experiment there were stated the development of chlamydospores with thick membranes of light-brown colour in the fungus colonies. Basing on the complex of morphological characteristics these strains of fungi were previously identified[14] as the representatives of *Fusarium* genus (figure 1).

Fig. 1. Morphological characteristics of colonies of strains 1c and 1y of the fungus of Fusarium genus (Strain colony 1c of the Fusarium fungus, which has derived from the invaded tissues of Ulmus Pumila; 2. Strain colony 1y of the Fusarium fungus in the pure culture).

The final identification of the 2 investigated strains of fungi was performed on the basis of PCR results. The results of the comparative analysis of nucleic acid sequences of ITS regions of the investigated strains and lodged in the international samples database GeneBank showed that strains 1c and 2y are the representatives of Fusarium genus:1) 2C – *Fusarium oxysporum*, which identity of nucleic acid sequence under the standard number EU625403.1 inGeneBankis 100%;2) 1A –*Fusarium solani*, which identity of nucleic acid sequence under the standard numbersFJ914886.1 and FJ478128.11 in GeneBank is 99,0%.

Discussion of the results of investigation

The results of our investigations showed that the fungus strains *Fusarium solani* and *Fusarium oxysporum*, which were derived from the invaded tissues of Ulmus Pumila are phytopathogens. This is proved by the results of investigation taken from the literature sources, in which it is stated that the most representatives of the *Fusarium* genus are the pathogens for different

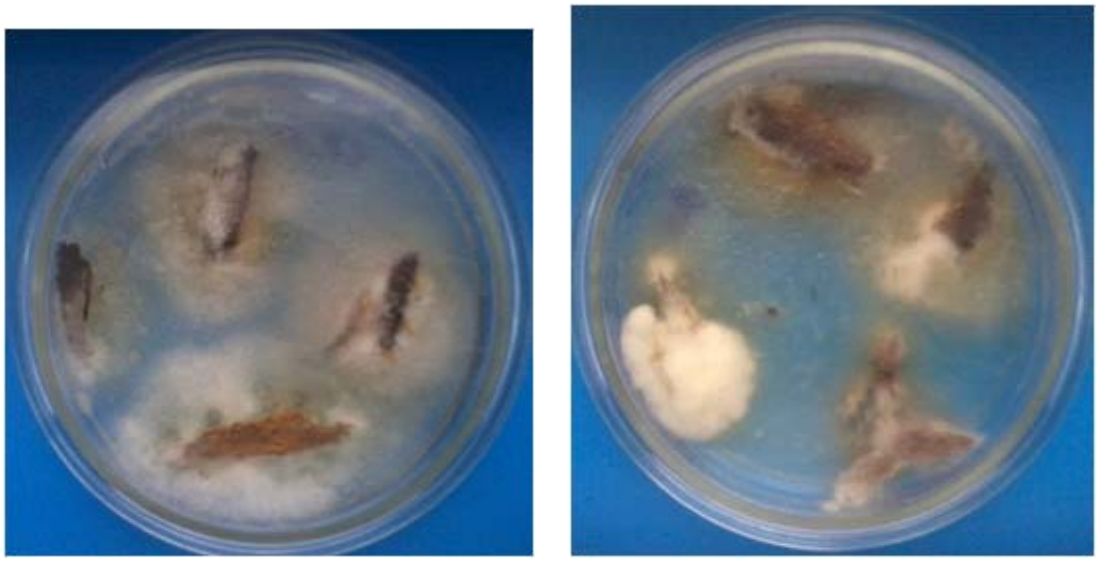

Fig. 1. Morphological characteristics of colonies of strains 1c and 1y of the fungus of Fusarium genus (Strain colony 1c of the Fusarium fungus, which has derived from the invaded tissues of Ulmus Pumila; 2. Strain colony 1y of the Fusarium fungus in the pure culture)

types of plants. Also we have found out that Fusariums of one type can invade plants of different families, causing various pathologies such as rot of roots, seeds, fruits as well as general inhibition and premature withering. Such representatives of this genus as *F. avenaceum, F. solani, F. culniorum, F. gibbosum, F. Semitectum* cause root rot of herbage plants such as potato, pea, pinto, melon, water-melon and tomato. The possibility of woody plants invasion by these fungi is proved by the results of many investigations. The investigated strains of fungi which "specialize" in conifers are *F. oxysporum, F. nivale*8 *F. Solani*[15, 16]. The invasion of other types of trees is proved by the similar results of study, made by the investigators from India. They stated that some types of Fusarium genus are harmful for 10 types of trees in the state of Punjub, India, and in Nepal.

70% of the forest stand died of the diseases, caused by these fungi. *Fusarium Solani* was recognised as the most harmful and aggressive one among the mentioned types of fungi[17-19]. Literary information about the other type of Fusarium– *Fusarium oxysporum* shows that it is the main pathogen of the tracheo-mycotic withering of plants. It is known that this disease can be found in regions with dry and hot summer.

Thus the results of our investigation prove that the studied strains of fungi have phytopathogenic features. Under the conditions of the arid climate of the Southern Kazakhstan these pathogens invade tissues of subcortial cortex of *Ulmus Pumila* stems. Invasive types of blasts, which larvas damage the stem wood and spread fungus spores contribute to the invasion of the adult trees by these pathogens. Invaded tissues and blasts excrements are the medium for the development of the secondary microbial flora, which together with the pathogens cause the rot of a plants stem. Due to this fact the ecological state of the Ulmus Pumila in dendroflora of Kazakhstan is nowadyas a serious problem. In order to solve it it is necessary to perform investigations aimed at finding out local strains of micro-organisms-antagonists- *F. oxysporum*8 *F. solani.* The prospects of the biological method of fighting Fusarium fungi is proved by the results of a lot of experiments with the use of biopesticides, developed on the basis of antagonistically active strains of fungi of Trichoderma genus (*Trichoderma asperelhtm*), actinomycetes of Streptomyces genus (*Streptomyces lateritius*) and bacteris of Bacilus genus (*Bacillus subtilis*), which are being successfully used for the protection of agricultural crops ad products after harvesting[20-22].

CONCLUSION

Thus, with the help of the results of the molecular-genetic analysis it was stated that the pathogens of invasive diseases of the Ulmus Pumila stems in Kazakhstan are microfungi of *Fusarium – F.solani* genus and *F.oxysporum* genus, which can be characterised as widely spread phytopathogens of a lot of types of herbage and woody plants. Nowadays the investigations are aimed at finding out the microorganisms-antagonists of these phytopathogens.

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