Molecular-genetic identification of emerged novel invasive pathogens of Asiatic Elm *Ulmus pumila L*

[1]M. Turaliyeva, [1]A.Yeshibaev, [2] I. Digel, [1]G. Elibayeva, [1]A. Sydykova, [1]A. Uspabayeva, [3]G. Dosybayeva, [1]A. Zhylysbayeva and [3]K. Lakhanova

[1] Department of Biotechnology, South Kazakhstan State University named after M. Auezov, Shymkent, Kazakhstan
[2] Institute of Bioengineering, FH Aachen University of Applied Sciences, Juelich, 52428, Germany
[3]Kh.Yassavi International Kazakh-Turkish University, Divaeva Str. 142/9, Shymkent, 160013, Kazakhstan

Abstract. The dwarf elm *Ulmus pumila* L. (Ulmaceae) is one of indigenous species of flora in Kazakhstan and forms a basis of dendroflora in virtually all settlements of the region. In the past decade, multiple outbreaks of previously unknown diseases of the small-leaved elm have been registered. In our study, by the molecular-genetic analysis it was found that the pathogens responsible for the outbreaks are microfungi belonging to the genus *Fusarium – F. solani* and *F. oxysporum.* The nucleotide sequences (ITS regions) isolated from the diseased trees showed very high similarity with the GenBank control numbers EU625403.1 and FJ478128.1 (100.0 and 99.0 % respectively). Oncoming research will focus on the search of natural microbial antagonists of the discovered phytopathogens.
[Turaliyeva M., Yeshibaev A., Digel I., Elibayeva G., Sydykova A., Uspabayeva A., Dosybayeva G., Zhylysbayeva A., Lakhanova K. **Molecular-genetic identification of emerged novel invasive pathogens of Asiatic Elm *Ulmus pumila L.*** *Life Sci J* 2014;11(5s):171-175]. (ISSN:1097-8135). http://www.lifesciencesite.com. 33

Keywords: Phytopathogen, dwarf elm, Fusarium, toxicity, micromycetes, antagonists, molecular-genetic analysis.

Introduction

Ulmus pumila L. (Ulmaceae) is one of indigenous species kazakhstanian flora which forms a basis of dendroflora in virtually all South-Kazakhstan settlements. According to researchers' information its share in wood of region is 37.8 % [1, 2]. *U. pumila* is notable for its high resistance to arid climate conditions that explains its wide prevalence in Central Asian region. For decades, there were no records or reports of novel epiphytotic diseases of *Ulmus pumila* in Kazakhstan. According to literature data, *U. pumila* is rather resistant to most diseases commonly affecting elms like the Dutch disease caused by Nectria fungi (*N. cinnabarina*) and Ophiostoma (*O. novo-ulmi* and *O. himal-ulmi*) which caused mass blight and extinguishment of whole elm populations in some European countries [3-6].

Outbreaks of novel, previously unknown diseases of trees *U. pumila* in Kazakhstan dendroflora became increasingly evident over the last 10 years. We suppose that this process was connected with invasion of ecdemic xilophagous pests such as *Monochamus urussovi* F., *Monochamus sutor* L., *Acanthocinusa edelis* L., *Cetonia aureta* L., *Cossus cossus, Xanthogaleruca luteola* and *Archips xylostena*. Larvae of these insects are the main distributors of phytopathogenic microflora spores. The new invasive diseases manifest themselves in *U. pumila* as trunk rotting and appearance of cancer tumors on trunks that ultimately leads to tree death [7, 8]. According to L. Serikbai, current degree of visible infestation of adult *U. pumila* trees by phytopathogenic microorganisms in Southern

Kazakhstan is 75-80 % and the further escalation could lead to dramatic changes in the natural and artificial dendroflora of the region [9]. Therefore accurate identification of the emerged novel pathogens of dwarf elm was regarded as an urgent and important task. Knowledge of the pathogen's nature and physiology would allow developing adequate measures for prevention and treatment of the disease outbreaks. Thus, the main object of this work was molecular-genetic identification of emerged novel invasive pathogens of dwarf elm.

Material and methods

Two strains of micromycetes (further referred to as 1c and 2y), were isolated from the diseased trunk *U. pumila* and used as the material for the further analysis (figure 1). The pure culture of the supposed phytopathogens was obtained by streak plate method on a selective nutrient Chapeka media under sterile working conditions. Isolation and count of micromycetes were carried out by conventional mycological methods [10-12].

Identification of the isolated microorganisms was carried out by direct determination of nucleotide sequences of their Internal transcribed spacer (ITS) regions and with the subsequent comparison of their identity with nucleotide sequences deposited in the international database (GenBank) of samples, and

also construction of phylogenetic trees using nucleotide sequences of referential strains.

Figure 1. Appearance of the diseased of dwarf elm trunks

DNA extraction and isolation was conducted in a DNA-extraction buffer (100 mMTris-HCl, 1,4 M NaCl, 20 mM EDTA, 2 % CTAB and K 100 µg/ml proteinase pH 8.0). The microbial culture, after centrifuging and removing of supernatant, was triturated pounder with addition of liquid nitrogen. Then in 100 µl of the obtained suspension were incubated for 18 hours, at a temperature of 37°C in the plastic tube in 500 µl of the extraction buffer. The suspension was then cleared by adding 750 µl of chloroform/isoamyl alcohol (24/1), subsequent stirring and centrifuging at 12 000 rpm for 10 minutes (Eppendorf 5418R). The water phase was then cleared repeatedly with phenol/chloroform isoamyl alcohol (24/24/1). DNA precipitation was conducted with 0.6 volumes of isopropyl alcohol followed by centrifugation at 37ºC for 2 hours. The DNA sediment was finally washed with 70 % ethyl alcohol under room temperature, with subsequent centrifuging 12,000 rpm for 10 minutes at 37ºC and removing of the liquid phase. The received DNA-sediment was dried in the open air for 15 minutes and then was dissolved in 100 µl of single buffer TE (TE x 1: Tris (2-amino-2-hydroxymethyl-1,3-propanediol) 10 mM 1 mM EDTA pH is not neutral and alkalescent (7.4 or 8.0)) and stored at 20°C. DNA concentration was measured spectrophotometerically (Nano Drop 2000 C, Thermo, USA) at 260 nm [13].

Amplification of the ITS region was conducted in PCR reaction with primers ITS 5 5 ' - ggaagtaaaagtcgtaacaagg-3 ' and ITS 4 5 ' - tcctccgcttattgatatgc-3' in total volume of 30 µl. [14]. PCR mixture contained 40 ng of DNA, 1unit of TaqDNAPolymerase (Fermentas), 0,2 mM of each deoxyonucleoside triphosphate, 1 PCR buffer (Fermentas) and 2,5 mMMgCl_2, per 10 pmol of each primer.

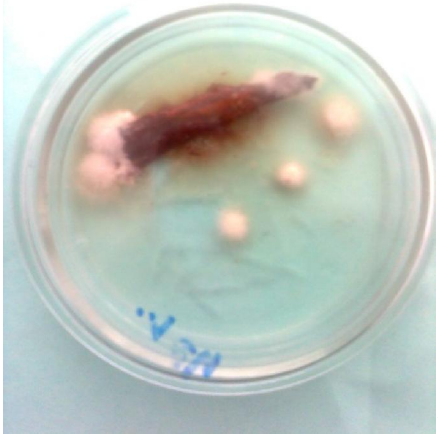
Program of PCR amplification included a long denaturation at 95°C for 4 minutes; 30 cycles, each consisting of following steps: 95°C – for 25 seconds, 52°C – for 30 seconds, 72°C – for 40 seconds. Final elongation was conducted at 72°C for 7 minutes. PCR program was carried out on amplifier DNAEngineTetrad 2 CyclerPTC-0240 (Bio-Rad). Before determination of PCR products nucleotide sequence was cleared from unconjugated primers using ExonucleaseI (Fermentas) and Alkaline Phosphatase, (Fermentas). Sequence reaction was conducted with application BigDye® Terminator v3.1 Cycle SequencingKit (Applide Biosystems), according to the instruction of the manufacturer, with the subsequent division of fragments with the automatic genetic analyzer 3730x l DNA Analyzer (Applide Biosystems).

Nucleotide sequences were analyzed and aligned in the general sequence using SeqMan (DNAStar) software. DNA sequences, after removal of final fragments of primers and fragments with low indicators of quality, were identified in GenBank according to BLAST algorithm [15].

Results

Examination of micro- and macro-morphological features of the isolated micromycetes in pure cultures showed that both samples belong to the class Deuteromycetes. These strains, with well-developed mycelia, formed on an agar nutrient media convex velvet colonies of pink color, 1.5-2.0 cm in diameter. Micro - and macro conidia of this fungus were distinctly bent, in 20-70 µm size, having various numbers of partitions with a distinct basal cell. Chlamydospore formation was observed on 25th day of culturing. Brown-colored chlamydospores were approximately 20 µm in diameter. On the basis of the above mentioned morphological features these strains were preliminary identified [16], as the representatives of *Fusarium* genus (Tuberculariaceae; Moniliales; Fungi imperfective) (figure 1).

Final identification of the isolated 2 fungi strains was conducted by molecular-genetic analysis. Comparison of nucleotide sequences of ITS regions from the isolated samples with sequences deposited in the international database GenBank (http://www.ncbi.nlm.nih.gov/genbank/) allowed to identify that 1c as *Fusarium solani* (FJ478128.11, 99.9% identity) and 2y as *Fusarium oxysporum* (EU625403.,100% identity).


1

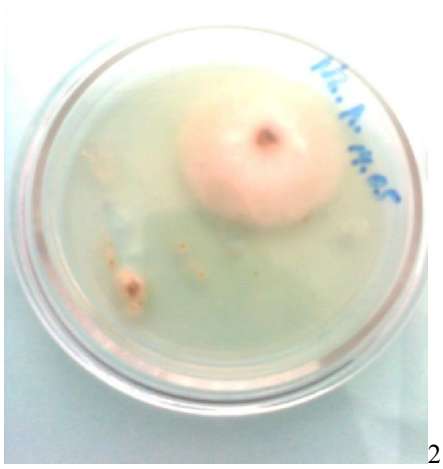

2

Figure 2. 1) Colones the 1c strain isolated from the diseased tissue of dwarf elm; 2) Colonies of the 1y strain in pure culture. Both samples were identified as *Fusarium* sp.

Discussion

Majority of *Fusarium* fungi are known agents of diseases of many various species and families of plants [17]. Fusariums damage plants, causing various pathological phenomena, such as rotting of roots, seeds, fruits, as well as general suppression and premature wilt. *F. avenaceum, F. solani, F. culniorum, F. gibbosum, F. semitectum, F. javanicum, F. heterosporum* are typically responsible for root rotting of potato, peas, kidney bean, melon, water-melon and tomato. *F. graminearum* and *F. nivale* are mainly agents of diseases of grains, casing vascular wilt and a snow mold [18-20].

Possibility of damage of also woody plants by *Fusarium* has been proved by many research groups. For example, Russian authors established high pathogenicity of *F.oxysporum, F.nivale* and *F. solani* for seedlings of conifers [20-26]. The strains studied so far appeared to be heterogeneous regarding to the degree of toxicity, virulence and pathogenicity. High degree of toxicity did not always correlate with high pathogenicity and virulence [27]. It was also revealed that some isolates can support existing infectious process, being low- or non-pathogenic themselves.

Indian researchers reported that in Nepal and Indian province of Punjab *Fusarium* species actively damaged ten species of trees. For example, from the diseases caused by these fungi, 70 % of forest *Dalbergia sissoo* Roxb was completely lost. Another Indian research group has distinguished *Fusarium solani* as the most harmful and aggressive.

Literature data on other fusarium species – *Fusarium oxysporum* suggests, that it is the main causative agent of tracheomycosis of plants [28-30], It is known, that this disease is mainly found in areas with dry and hot summer. Most typical for this disease is a droop of plant crown, loss of leaves turgor, their yellowing, wilt and full shrinkage. Such disease is marked on banana plants (*Musabalbisiana* Colla), silk tree *(Albiziajulibrissin* Durz) and ailanthus (*Ailantusaltissima*), at which *F. oxysporum* colonizes and blocks up vascular tissue and obstructs with juice movement on spending systems owing to which plants quickly perish [31-33].

Thus, results support the existing data and contribute a new evidence of wide spread and broad spectrum of pathogenic properties of *Fusarium* species on trees. In conditions of arid climate in Southern Kazakhstan these pathogens damage mainly the subcortical bast in dwarf elm trees. To damage of adult trees by these pathogens promote parasitic activity of insect pests' larvae which damage wood and spread spores of the fungi. The affected tissue and insects' excrements provide a medium for development of the secondary microflora, facilitating the tree damage and death. In this connection, in the present time ecological condition of dwarf elm in Kazakhstan dendroflora is the serious problem which solution demands urgent measures on revealing of local natural antagonistic microorganisms in respect to *F. oxysporum* and *F. solani.* This would represent probably the most advanced method of fusarial fungi control, which was suggested by many previous successful attempts reported elsewhere [34-37].The biopesticides developed on a basis antagonistic microbial strains of fungi (*Trichoderma asperelhtm*), ray fungi (*Streptomyces lateritius*) and bacteria (*Bacillus subtilis*) are currently successfully used for protection of agricultural before and after harvesting.

Conclusion

By combining morphological and molecular-genetic analysis we have identified the causative agents of recent outbreaks of novel infectious disease of dwarf elm as two species of *Fusarium* - *F.solani* and *F.oxysporum*. These agents significantly

endanger the existing population of dwarf elm trees in Southern Kazakhstan. The nucleotide sequences (ITS regions) isolated from the diseased trees showed very high similarity with the GenBank control numbers EU625403.1 and FJ478128.1 (100.0 and 99.0 %, respectively). Oncoming research should focus on the search of natural microbial antagonists of the discovered phytopathogens.

Corresponding Author:
Dr. M. Turaliyeva
Department of Biotechnology, South Kazakhstan State University named after M. Auezov, Shymkent, Kazakhstan

References

1. Sokolov, S.J., 1951. Trees and bushes of the USSR. Wild-growing, cultivated and perspective for introduction. Ulmus L. - Elm, or smooth-leaved elm. Moscow: Publ AS of the USSR, pp: 509-510.
2. Sikhymbaev, A.E., K.T. Kerimbekova and M.S. Sikhimbaeva, 2005. Alien plants in Southern Kazakhstan. Shymkent: Educational book, pp: 31-34.
3. Smalley, E. and R.P. Guries, 2005. Breeding elms for resistance to Dutch elm disease. Annual Review of Phytopathology, 31: 25-35.
4. Sidorova, I.I., 1991. Group of orders Plektomyces. Order of microascovy. Ofistoma. elm. M: Education, pp: 125-126.
5. Dudina, V.S., 1938. The Dutch disease of elms. M: Nauka, pp: 46.
6. The Dutch disease of elms. Forestry Encyclopedia: in 2 volumes, 2006. M: VNIILM, pp: 155-156.
7. Nurtazaeva, N., 2011. Influence of strengthening of urbanization process on an ecological condition of dendroflora. Materials of International Conference exhibition Science, technics and innovative technologies during an epoch of great Renaissance. Ashkhabad, 8: 189-193.
8. Nurtazaev, N. and S. Aleuova, 2010. Results of biology research of pest's development as Monochamusurussovi and Xanthogalerucaluteola. Materials of the International Research and Practice Conference "Problems of modern science and education", Shymkent, pp: 439-443.
9. Serikbay, L., 2011. Evaluation of phytosanitary conditions of flora in Shymkent city. Republican Students' Conference, Shymkent, pp: 216.
10. Dudka, I.A., 1982. Methods of experimental mycology. Kiev, pp: 438.
11. Methods of soil microbiology and biochemistry, 1991. M: Moscow State University Publishing house, pp: 304.
12. Tepper, E.Z., K.V. Shilnikova and G.I. Pereverzeva, 2004. A practical work on microbiology. M: Dropha, pp: 256.
13. Lluelin, M.B., 1999. Determination of DNA nucleotide sequences. Molecular clinical diagnostics. Methods. M: Mir, pp: 428 - 447.
14. Vegas E. Z. S., Nieves B., Araque M., Velasco E., Ruiz J., Vila J. Outbreak of Infection With Acinetobacter Strain RUH 1139 in an Intensive Care Unit, Infection control and hospital epidemiology. – 2006. - Vol. 27, № 4. - pp: 397 – 404.
15. Werle E., Schneider C., Renner M., Völker M., Fiehn W. Convenient single-step, one tube purification of PCR products for direct sequencing, Nucleic Acids Res. – 1994, Vol. 22, pp. 4354-4355.
16. Satton, D., A. Fotergill and M. Rinaldi, 2001. Determinant of pathogenic and conditionally pathogenic fungi. Mir, pp: 48-56.
17. Root decays of spring wheat, 1974. Leningrad: Under S.M. Tupenevich's edition, pp: 1-64.
18. Instructions on protection of grain cereals from root decays, 1978. Moscow: the Ministry of Agriculture of the USSR, pp: 1-28.
19. Benken, A.A., L.K. Hatskevich and A. Nesterov, 1987. Problem of root decay of cereals. Mycology and Phytopathology, 6(21): 566-573.
20. Babajants, L.T. and E.A. Klechkovskaya, 1988. Estimation of stability of wheat to fusarial decay. Methodical instructions. Odessa, pp: 1-20.
21. Jones, J.P., and Woltz, S.S. 1981. Fusarium-incited diseases of tomato and potato and their control in Fusarium: Diseases, Biology, and Taxonomy. pp: 157-168
22. Kvashnina, E.S., 1976. Physiological and ecological features of species as Fusarium of Sporotrichiella. Mycology and phytopathology, 4(10): 275-282.
23. Kvashnina, E.S., 1978. Morphological and cultural properties of species as Fusarium of Sporotrichiella and their natural habitat in the USSR. Mycology and phytopathology, 1: 3-10.
24. Kiviniemi, S.N., 1990. Fusariose in nursery forest of Kareliya. Protection of nurseries and underwoods against pests and diseases: thesis report in All-Union scientific and tech. meeting, Nauka, 10: 33-35.
25. Kuznetsov, A.I., 1991. Seedling lodging of conifers in the conditions of Murmansk area and control measure of it. Microbiological and

phytopathological researches in the Kola North, 2: 87-91.
26. Nozdrenko, Ja.V., 1990. Major fungus diseases of conifers seedling in wood nurseries of Novosibirsk area. Protection of nurseries and underwoods against pests and diseases: thesis report in All-Union scientific and tech. meeting, Nauka, pp: 68-69.
27. Pak. J. Bot., 2008. Pathogenicity and host range of Fusarium solani (mart.) Sacc.causing dieback of Shisham (DalbergiasissooRoxb.), 40 (6): 2631-2639.
28. Javaid, A., R. Bajwa and A. Tahmina. 2004. Tree dieback in Punjab, Pakistan. Mycopath, 2: 1-5.
29. Khan, M.M., T. Mahmood and R.M. Rafique. 1999, Diagnostic study of shisham dieback in Punjab. Forestry Research Institute, Faisalabad. Proc. of 2nd Natl. Conf. of Plant Pathol. Univ. of Agri. Faisalabad, pp: 15-19.
30. Khan, M.M. and M.H. Khan, 2000. Dieback of Dalbergiasissoo Roxb. in Pakistan. In: Proc. of the Sub-Regional seminar on dieback of sissoo (Dalbergiasissoo Roxb.), Katmandu, Nepal, pp. 51-56.
31. Ploetz RC and Pegg KG, 2000. Fusarium wilt. In Jones DR (ed.) Diseases of banana and tabaca, CABI, Wallingford, UK. pp: 143-159.
32. Stipes, R. J. 1999. Fusarium wilt of trees, Shade Tree Wilt Diseases. C. L. Ash, ed. APS Press, St. Paul, MN. pp: 165-169
33. Bush E, 2008. Fusarium Wilt of Mimosa (Albiziajulibrissin). Department of Plant Pathology, Physiology, and Weed Science. Virginia co-operative Extension. www.ext.vt.edu <http://www.ext.vt.edu>
34. Cheatham S, M. Johnston and L, Marshall, 1996. Albizia. In: The Useful Wild Plants of Texas, the Southeastern and Southwestern United States, the Southern plains, and Northern Mexico. Useful Wild Plants Inc., TX, pp: 188-190.
35. Panahian, Gh. And K. Rahnama, 2010. Fusarium wilts on indigenous silk trees (Albiziajulibrissin Durz) in the north ofIran, Gorgan. International journal of Agronomy and Plant Production. 1 (1): 1-5.
36. Aleksandrova, A.V. and L.L. Velikanov, 2000. Influence of fungus as Trichodennaharzianum on soil micromycetes. Mycology and phytopathology, 3: 68-77.
37. Gromovyh, T.N., M.V. Gukaejan, L.A. Malinovsky, A.V. Tjulpanova and T.I. Golovanova, 1997. Use of microbic antagonism in control of infectious lodging of conifer seedling. The Siberian ecological journal, 5: 501-504.

3/23/2014