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SZENT ISTVÁN UNIVERSITY

The Faculty of Horticulture

IDENTIFICATION AND INVESTIGATION OF PHYTOPLASMAS OCCURING IN HUNGARY BY MOLECULAR METHODS

Theses of doctoral dissertation

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2. **Viczián, O. and Süle, S**. PCR identification of pitted fruit tree phytoplasmas. Presented at the 42nd Plant Protection Days, Budapest, 1996.
3. **Viczián, O. and Süle, S.** Phytoplasma decline of apricot in Hungary. Presented at the "Lippay János" scientific sessions, Budapest, 1996.
4. **Viczián, O., Süle, S. and Gáborjányi, R.** Questions concerning the epidemiology of the grape stolbur disease. Presented at the session on the ”Integrated Plant Protection of Grape” of the plant health and horticultural working committee of the Pécs academic committee, Pécs, 1998.
5. **Viczián, O., Süle, S. and Gáborjányi, R.** Phytoplasma diseases in Hungary. Presented at the 44th Plant Protection Days, Budapest, 1998.
6. **Viczián, O., Süle, S. and Gáborjányi, R.** Natural plant hosts of the stolbur phytoplasma in Hungary. Presented at the "Lippay János-Vas Károly" scientific sessions, Budapest, 1998.

As yet, very little is known about phytoplasma pathogenicity. Increased understanding of the pathogen will help improve the techniques used to protect plants from the disease; as such, we attempted to identify and analyse certain phytoplasma genes. Because of the frequency of the disease an easy-to-use and modestly priced ELISA test needs to be developed. The ELISA test would be an efficient tool for the larger-scale testing of plant materials brought in Hungary and plant material already in Hungary, thereby increasing the possibility to screen out infected material and limit the spread of the disease.
Decrease in the use of pesticides over the last decade has contributed to an increase in the rate of infection with the pathogen oftentimes over-wintering in neighbouring weeds which thereby become a source for infection the following year. As such, we intend to increase our testing of weeds.

3. Materials and methods

Plant materials: DNA samples from standard phytoplasma groups (AAY, ESFY, AP and STO-stolbur) extracted from periwinkle and tobacco plants during our stay in Germany were used as positive controls. In Germany, these phytoplasmas were maintained through grafting in a greenhouse; these same methods were used by us in Hungary. Plant materials that were tested were brought to us from various points throughout the country. A stolbur strain that we collected was maintained through grafting on tomato.

Detection and identification: Phytoplasma infections can be detected both "directly" and "indirectly". For direct detection, epifluorescent DAPI staining was used; for the most part, however, the much more sensitive PCR technique (both simple and nested) was used. Hybridization was also attempted. Different extraction techniques were tried in order to obtain as pure a DNA sample as possible. For PCR both universal and group-specific primer pairs were used.
RFLP was used to identify unknown strains. RFLP results were compared to data in the literature and strains were identified as members of specific phytoplasma groups.

Searching for phytoplasma genes: Phytoplasma genes were sought in an AP phytoplasma DNA λ-phage library. Using a phytoplasma-enrichment technique, we extracted proteins from AP-infected periwinkle and tobacco. Diagnosticum KFT used the protein extract to produce, in mice, antiserum containing both polyclonal and monoclonal antibodies. The antiserum was used for the immunoblot library-screening.
Cloning of a known phytoplasma gene: We are preparing antiserum for the *tuf* gene of the AP phytoplasma for use in ELISA tests. As a first step the gene was inserted into a AT-cloning plasmid. The gene was then amplified with primers we designed containing known restriction sites; this allowed for the gene to be subcloned into an expression vector with the sequence in the proper direction and reading-frame. The gene-product was then expressed; and we are now attempting to isolate it.

4. Results and discussion:

Symptoms of phytoplasma infection: Over the past few years, plants exhibiting typical symptoms such as yellowing and witches' broom were observed on numerous occasions in both orchards and vegetable cultures. Oftentimes the altered shape and colour of the flower hinted at an unusual disease. In certain cases, symptoms did not necessarily imply phytoplasma infection with many other pathogens and nutrient deficiencies known to cause yellowing (e.g. in apricot, pepper and Japanese plum). In other cases, (e.g. apple, tomato, parsley and celery), on the basis of symptoms alone we could be sure that a phytoplasma infection had occurred. However we should caution that phytoplasma infections cannot be diagnosed based on symptoms alone.

Detection and identification: Detection was successful using both "direct" and "indirect" techniques. The direct epifluorescence technique gave results only when the phytoplasmas were present at a high concentration; thus, when taking into account the rate of spreading of the disease, this technique proved inadequate. The hybridization technique, although it was able to detect phytoplasma infections at lower concentrations, is both expensive and time-consuming, being thereby unsuitable for larger-scale testing. PCR proved to be the most sensitive technique; and digestion with restriction enzymes (RFLP) of the PCR amplified product was used to identify specific strains.

Phytoplasma infections occurring in Hungary and their plant hosts: As yet, phytoplasma strains from 5 groups (AAY, ESFY, AP, CPh and Stolbur) have been identified on 24 plant species. We identified the first ESFY infections in Hungary of apricot, Japanese plum, almond and cherry, AP infection of apple, AAY infections of *Fagopyrum dumetorum*, cabbage, red cabbage, Brussels sprouts, kale and cauliflower, stolbur infections of celery, parsley, carrot, bladder campion, tobacco, jimsonweed, rapeseed, grape and dandelion, and CPh infection of white clover.
The above-mentioned results are the first report of stolbur infections of *Fagopyrum dumetorum*, bladder campion and dandelion.

No reports could be found in either the Hungarian or international literature on the phytoplasma infection in daisy that we identified; however we have, as yet, been unable to determine which group the strain belongs to.

Our plant material samples originate from the Békés, Csongrád, Heves, Pest, Szabolcs-Szatmár-Bereg and Zala counties. In Békés and Csongrád, the stolbur infections of pepper, tomato, parsley, celery and carrot were collected. In Heves county a high rate of stolbur infection was generally observed in open-air pepper and tomato cultures, with certain pepper cultures, both covered and open-air, having infection rates as high as 95%. In Pest county we detected AAY infections on Brussels sprouts, cabbage, cauliflower, kale, *Fagopyrum dumetorum* and red cabbage, as well as the, as yet, unknown phytoplasma infection of daisy. In the Szabolcs-Szatmár-Bereg county a stolbur infection was detected on tobacco. In Zala county stolbur infections were identified on jimsonweed, dandelion, tomato, pepper, rapeseed, bladder campion and grape, an ESFY infection on almond, and a CPh infection on white clover.

Searching for phytoplasma genes: For the immunization, by combining several protein purification techniques, we worked out a technique that yielded a sufficient amount of purified protein. Through immunoblot screening of the AP λ-phage library with the polyclonal antiserum obtained through immunization, we isolated a 50 bp sequence and a 100 bp sequence of AP phytoplasma DNA. The short length of the isolated inserts implied that it was highly unlikely that the full sequence of their respective open-reading frames had been isolated; they were therefore considered inappropriate for antiserum production.

Cloning of the AP *tuf* gene: An amplified sequence containing the *tuf* gene was cloned into an AT plasmid; the inserted gene was then successfully amplified using primers we designed containing known restriction sites. The gene was cloned with its sequence in the proper direction and reading-frame into an expression vector with its own regulatory promoter.
Following the cloning of the gene, its protein was expressed and separated/isolated using PAGE.

We are currently working on increasing the quantity of antigen produced to a level sufficient for immunization, thus eventually allowing for the development of a serological test for phytoplasmas.

5. Publications relevant to the PhD defence
Publications relevant to the thesis:
Articles:

1. Previous research

Phytoplasmas, previously known as MLOs or mycoplasmalike organisms, are wall-less plant pathological prokaryotes that do not grow on any known culture media. Since the 1960s, known symptoms of phytoplasma infection including yellowing, stunting and witches' broom have been observed on hundreds of plants. Their taxonomic position has yet to be fully elucidated; however classification is now far more accessible thanks to improvements in molecular techniques over the past few decades that today allow for its widespread application. Immunological and genetic analysis have conformed that phytoplasmas belong to the *Mollicutes* class; however they are only distantly related to mycoplasmas. The organisms most closely related to them are from the genus *Acholeplasma*. Consequently in 1994, mycoplasmalike organisms were renamed phytoplasmas, clearly reflecting their role in plant pathogenicity.

Within plants, phytoplasmas are only known to live and multiply within phloem sieve-tube cells. Following phytoplasma infections, Callose and starch accumulations can be observed at collapsed sieve-tube cells. The decrease in the flow of sap results in nutrient deficiencies within plant tissues and a hormone imbalance. These, in turn, can cause serious disruptions in the development of both vegetative and flowering organs. Currently, there are three known methods for the transfer of phytoplasma from an infected plant to a healthy plant: via a dodder bridge, through leafhopper infection and by grafting. Any attempt to transfer the phytoplasma infection mechanically has failed.
Using modern genetic techniques (hybridisation, PCR, RFLP and sequence analysis), phytoplasma infections can be detected even when the pathogen is present at only very low concentrations. Very little is known about phytoplasma physiology and the exact mechanism whereby phytoplasmas cause their plant pathogenic symptoms. Using the above-mentioned techniques we have started to analyse phytoplasma genes in the hopes of answering certain questions about the organism.

2. Goals
Our first aim was to detect and classify phytoplasma infections throughout Hungary using the most current molecular techniques. We used these molecular techniques to reassess data on earlier phytoplasma infections detected using non-molecular techniques, and to confirm or refute phytoplasma infections in plants showing symptoms of the disease. The range of known plant hosts and regions afflicted with the disease within Hungary was also compared to earlier data.